

(19) World Intellectual Property
Organization
International Bureau



(43) International Publication Date
26 February 2004 (26.02.2004)

PCT

(10) International Publication Number
WO 2004/016282 A1

(51) International Patent Classification⁷: **A61K 39/385**,
39/00, A61P 25/00

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(21) International Application Number:
PCT/EP2003/007864

(22) International Filing Date: 18 July 2003 (18.07.2003)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
60/396,639 19 July 2002 (19.07.2002) US
60/470,432 15 May 2003 (15.05.2003) US

(81) Designated States (*national*): AE, AG, AL, AM, AT, AU,
AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CO, CR, CU,
CZ, DE, DK, DM, DZ, EC, EE, ES, FI, GB, GD, GE, GH,
GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC,
LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW,
MX, MZ, NI, NO, NZ, OM, PG, PH, PL, PT, RO, RU, SC,
SD, SE, SG, SK, SL, SY, TJ, TM, TN, TR, TT, TZ, UA,
UG, US, UZ, VC, VN, YU, ZA, ZM, ZW.

(84) Designated States (*regional*): ARIPO patent (GH, GM,
KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZM, ZW),
Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM),
European patent (AT, BE, BG, CH, CY, CZ, DE, DK, EE,
ES, FI, FR, GB, GR, HU, IE, IT, LU, MC, NL, PT, RO,
SE, SI, SK, TR), OAPI patent (BF, BJ, CF, CG, CI, CM,
GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

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Published:

- with international search report
- before the expiration of the time limit for amending the
claims and to be republished in the event of receipt of
amendments

*For two-letter codes and other abbreviations, refer to the "Guid-
ance Notes on Codes and Abbreviations" appearing at the begin-
ning of each regular issue of the PCT Gazette.*

(54) Title: VACCINE COMPOSITIONS CONTAINING AMYLOID BETA1-6 ANTIGEN ARRAYS

(57) Abstract: The present invention is related to the fields of molecular biology, virology, immunology and medicine. The inven-
tion provides a composition comprising an ordered and repetitive antigen or antigenic determinant array, and in particular an A β 1-6
peptide-VLP-composition. More specifically, the invention provides a composition comprising a virus-like particle and at least one
A β 1-6 peptide bound thereto. The invention also provides a process for producing the conjugates and the ordered and repetitive
arrays, respectively. The compositions of the invention are useful in the production of vaccines for the treatment of Alzheimer's
disease and as a pharmaccine to prevent or cure Alzheimer's disease and to efficiently induce immune responses, in particular an-
tibody responses. Furthermore, the compositions of the invention are particularly useful to efficiently induce self-specific immune
responses within the indicated context.

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VACCINE COMPOSITIONS CONTAINING AMYLOID BETA1-6 ANTIGEN ARRAYS

BACKGROUND OF THE INVENTION

Field of the Invention

5 The present invention is related to the fields of molecular biology, virology, immunology and medicine. The invention provides a composition comprising an ordered and repetitive antigen or antigenic determinant array, and in particular an A β 1-6 peptide-VLP-composition. More specifically, the invention provides a composition comprising a virus-like particle and at least one A β 1-6 peptide bound
10 thereto. The invention also provides a process for producing the conjugates and the ordered and repetitive arrays, respectively. The compositions of the invention are useful in the production of vaccines for the treatment of Alzheimer's disease and as a pharmaccine to prevent or cure Alzheimer's disease and to efficiently induce immune responses, in particular antibody responses. Furthermore, the compositions
15 of the invention are particularly useful to efficiently induce self-specific immune responses within the indicated context.

Related Art

Alzheimer's disease (AD) is the most common cause of dementia among the elderly (age 65 and older) and a serious burden for public health. For example, 4
20 million people are reported to suffer from the disease in the United States of America. The incidence of the disease is expected to increase as the population ages.

The main pathological signs of Alzheimer's disease are age-related changes in behaviour, deposition of β -amyloid into insoluble plaques, called the neuritic plaques or AD plaques, neurofibrillary tangles composed of tau protein within neurons, and
25 loss of neurons throughout the forebrain. In addition to the late onset AD, which occurs in old age (65 years and more), there is an early onset AD, familial AD (FAD) occurring between age 35 and 60. The pathological abnormalities of AD are more widespread, severe and occur earlier in FAD than in late onset or sporadic AD.

Mutations in the APP gene, the presenilin 1 and the presenilin 2 genes have been correlated with FAD.

As indicated, one of the key events in Alzheimer's Disease (AD) is the deposition of amyloid as insoluble fibrous masses (amyloidogenesis) resulting in
5 extracellular neuritic plaques and deposits around the walls of cerebral blood vessels (for review see Selkoe, D. J. (1999) *Nature*. 399, A23-31). The major constituent of the neuritic plaques and congophilic angiopathy is amyloid β ($A\beta$), although these deposits also contain other proteins such as glycosaminoglycans and apolipoproteins. $A\beta$ is proteolytically cleaved from a much larger glycoprotein known as Amyloid
10 Precursor Protein (APP), which comprises isoforms of 695-770 amino acids with a single hydrophobic transmembrane region. $A\beta$ forms a group of peptides up to 43 amino acids in length showing considerable amino- and carboxy-terminal heterogeneity (truncation) as well as modifications (Roher, A. E., Palmer, K. C., Chau, V., & Ball, M. J. (1988) *J. Cell Biol.* 107, 2703-2716. Roher, A. E., Palmer, K.
15 C., Yurewicz, E. C., Ball, M. J., & Greenberg, B. D. (1993) *J. Neurochem.* 61, 1916-1926). Prominent isoforms are $A\beta$ 1-40 and 1-42. It has a high propensity to form β -sheets aggregating into fibrils, which ultimately leads to the amyloid.

$A\beta$ peptide has a central role in the neuropathology of Alzheimers disease. Region specific, extracellular accumulation of $A\beta$ peptide is accompanied by
20 microgliosis, cytoskeletal changes, dystrophic neuritis and synaptic loss. These pathological alterations are thought to be linked to the cognitive decline that defines the disease.

Administration of amyloid beta protein or, in particular, $A\beta$ 1-28 in amounts of up to 10^{-2} mg/dose in the absence of any adjuvants and without any linkage of the
25 amyloid beta protein or $A\beta$ 1-28 to a carrier, for the treatment of Alzheimer's disease, is described in EP 526'511.

Others have used administration of $A\beta$ peptides in combination with adjuvants, to induce an immune response, cellular or humoral, against $A\beta$ 1-42. In a transgenic mouse model of Alzheimer disease, animals overexpress human amyloid precursor
30 protein containing the mutation APP(717)V-F (PDAPP-mice; Johnson-Wood, K. *et al.*, *Proc. Natl. Acad. USA* 94: 1550-1555, Games, D. *et al.*, *Nature* 373: 523-527 (1995a)), leading to overproduction of $A\beta$ 1-42, develop plaques, dystrophic neuritis,

loss of presynaptic terminals, astrocytosis and microgliosis. In a recent study, Schenk, D. *et al.*, (*Nature* 400:173-77 (1999) and WO 99/27944) report that administration of aggregated A β ₁₋₄₂ mixed with a strong adjuvant (CFA/IFA), which cannot be used in humans, in the first 4 immunizations, followed by administration
5 of aggregated A β ₁₋₄₂ in PBS in the subsequent immunizations, to PDAPP-mice at 6 weeks of age, essentially prevented plaque formation and associated dystrophic neuritis. The same authors reported that immunization of older mice (11 months of age) using the same strategy markedly reduced the extent and progression of Alzheimer's disease (AD)-like neuropathologies. Proliferation of splenocytes from
10 mice immunized using the abovementioned strategy was reported in Example III (Screen for therapeutic Efficacy against established AD) of WO 99/27944, showing that A β ₁₋₄₂ specific T-cells were induced by the vaccination procedure. Coupling of A β fragments to sheep anti-mouse IgG, and immunization of said coupled fragment in the presence of the adjuvant CFA/IFA is reported in WO 99/27944. The use of
15 compositions comprising A β fragments linked to polypeptides such as diphtheria toxin for promoting an immune response against A β is also disclosed in WO 99/27944. However, no data of immunization are provided.

A monoclonal antibody recognizing an epitope within the N-terminus (1-16) of A β (antibody 6C6) has been shown to protect PC12 cells from neurotoxicity of
20 fibrillar β -amyloid, and to disaggregate β -amyloid in vitro (Solomon B. *et al.*, *Proc. Natl. Acad. Sci. USA* (1997)). A monoclonal antibody raised against A β ₁₋₂₈, was also shown to suppress β -amyloid aggregation in vitro (Solomon B. *et al.*, *Proc. Natl. Acad. Sci. USA* (1996)). Frenkel *et al.*, (*J. Neuroimmunol.* 88: 85-90 (1998)) have later identified the epitope of two anti-aggregating antibodies, 10D5 and 6C6,
25 as being the epitope "EFRH", i.e. A β ₃₋₆. In contrast, an antibody specific for A β ₁₋₇ was unable to prevent β -amyloid aggregation (Frenkel D. *et al.*, *J. Neuroimmunol.* 95: 136-142 (1999)).

A β ₁₋₄₂ is fibrillogenic, and indeed, the vaccine composition described in WO 99/27944 used A β ₁₋₄₂ treated in such a way that it can form aggregates. It has been
30 shown that those fibrils are toxic for neuronal cell cultures (Yankner *et al.*, *Science* 245: 417-420 (1989)), and that a toxic effect is also observed when injected into animal brains (Sigurdson *et al.*, *Neurobiol. Aging* 17: 893-901 (1996); Sigurdson *et*

al., *Neurobiol. Aging* 18: 591-608 (1997)). Walsh et al., (*Nature* 416:535-539 (2002)) report that natural oligomers of A β are formed within intracellular vesicles. Those oligomers inhibited long term potentiation in rats in vivo and disrupted synaptic plasticity at concentrations found in human brain and cerebrospinal fluid.

5 In another study, Bard, F. *et al.* (*Nature Medicine* 6:916-19 (2000)) reported that peripheral administration of antibodies raised against A β ₁₋₄₂, was able to reduce amyloid burden, despite relatively modest serum levels. This study utilized either polyclonal antibodies raised against A β ₁₋₄₂, or monoclonal antibodies raised against synthetic fragments derived from different regions of A β . Thus induction of
10 antibodies against A β peptides bears promises as a potential therapeutic treatment for Alzheimer disease.

Mucosal administration of an antigen associated with β -amyloid plaques, such as β -amyloid peptide and A β ₁₋₄₀, has been described in WO99/27949. Mucosal administration is said to suppress certain cytokine responses associated with
15 Alzheimer's disease, and to enhance certain other cytokine responses associated with the suppression of inflammatory responses linked to the disease. It is thought that suppression of the inflammatory responses is effected by the "elicitation of T-cells characterized by an anti-inflammatory cytokine profile". Suitable antigens, as described in WO99/27949, include antigens specific for AD, and which are
20 recognized by immune T-cells of a human or animal host.

Fusion of epitopes of a monoclonal antibody recognizing A β to coat proteins of filamentous phages is described in WO 01/18169. Immunization of mice with the filamentous phages displaying the 15-mer epitope VHEPHEFRHVALNPV (SEQ ID NO: 89) on the coat protein VIII resulted in antibodies recognizing A β 1-16, and
25 A β ₁₋₄₀. This was demonstrated in an inhibition ELISA using A β peptides, and an IC₅₀ of 1 μ M was found for inhibition of the binding of the sera to A β ₁₋₁₆ with A β ₁₋₄₀. Solomon (WO 01/18169), however, provides no indication that the sera elicited against the filamentous phages carrying the VHEPHEFRHVALNPV epitope (SEQ ID NO: 89), bind to amyloid plaques or neuritic plaques of AD.

30 There are a number of drawbacks in using sequences differing from the antigen against which an immune response is to be elicited for immunization. First,

antibodies against part of the sequence foreign to the antigen or antigenic determinant may be induced. Second, the conformation of the antigen in the context of the foreign flanking sequence element may be different than in the context of the full-length antigen. Thus, although antibodies cross-reacting to the antigen may be elicited, their binding to the antigen may be suboptimal. The fine specificity of those elicited antibody may also not correspond to the specificity of antibodies elicited against the antigen itself, as additional side-chains different from the residues present on the full-length A β are present in the epitope. Finally, a 15-mer amino-acid sequence may contain T-cell epitopes. Display of the epitope YYEFRH (SEQ ID NO: 90) on the protein III of filamentous phage coat, of which 3-5 copies only are usually present on each phage, is also disclosed in WO 01/18169. Several problems arise when using infectious phages as carrier for immunization. First, production of infectious agents in large scale and in sufficient quantity for large immunization campaigns is problematic. Second, the presence of the DNA of the phage containing antibiotic resistance genes in the vaccine is not unproblematic and is a safety issue. Finally, the feasibility and efficacy of irradiation of large quantities of phages, in the case where non-infectious phages are used as vaccine, is unresolved.

A β analogues, wherein A β is modified to include T helper epitopes have been described (WO 01/62284). Immunization of TgRND8+ mice, transgenic for human APP, with the A β analogue resulted in a 4- to 7.5-fold higher antibody titer over immunization with A β 1-42 in the absence of adjuvant.

Recent studies demonstrated that a vaccination-induced reduction in brain amyloid deposits has the potential to improve cognitive functions (Schenk, D., *et al. Nature* 400: 173-177 (1999); Janus, C. *et al., Nature* 408: 979-982 (2000); Morgan, D. *et al., Nature* 408 : 982-985 (2000)).

The autopsy of a patient immunised with aggregated A β 1-42 in the Adjuvant QS21 has revealed the presence of a T-lymphocyte meningoencephalitis and infiltration of cerebral white matter by macrophages (Nicoll, J.A. *et al., Nature Med.* 9: 448-452 (2003)).

Recently, a publication has reported 18 cases of meningoencephalitis in patients immunized by the AN1792, a vaccine composed of aggregated A β 1-42 and QS-21 as adjuvant (Orgogozo J.-M. *et al., Neurology* 61: 46-54 (2003)). T-cell

activation is reported as a potential mechanism responsible for the disease, while there was no clear relation between disease and anti-A β 1-42 titers in the serum.

It is well established that the administration of purified proteins alone is usually not sufficient to elicit a strong immune response; isolated antigen generally must be
5 given together with helper substances called adjuvants. Within these adjuvants, the administered antigen is protected against rapid degradation, and the adjuvant provides an extended release of a low level of antigen. In the present invention, A β peptides are made immunogenic through binding to a VLP and do not require an adjuvant.

10 One way to improve the efficiency of vaccination is thus to increase the degree of repetitiveness of the antigen applied. Unlike isolated proteins, viruses induce prompt and efficient immune responses in the absence of any adjuvants both with and without T-cell help (Bachmann and Zinkernagel, *Ann. Rev. Immunol.* 15:235-270 (1991)). Although viruses often consist of few proteins, they are able to trigger
15 much stronger immune responses than their isolated components. For B-cell responses, it is known that one crucial factor for the immunogenicity of viruses is the repetitiveness and order of surface epitopes. Many viruses exhibit a quasi-crystalline surface that displays a regular array of epitopes which efficiently crosslinks epitope-specific immunoglobulins on B cells (Bachmann and Zinkernagel, *Immunol. Today*
20 17:553-558 (1996)). This crosslinking of surface immunoglobulins on B cells is a strong activation signal that directly induces cell-cycle progression and the production of IgM antibodies. Further, such triggered B cells are able to activate T helper cells, which in turn induce a switch from IgM to IgG antibody production in B cells and the generation of long-lived B cell memory - the goal of any vaccination
25 (Bachmann and Zinkernagel, *Ann. Rev. Immunol.* 15:235-270 (1997)). Viral structure is even linked to the generation of anti-antibodies in autoimmune disease and as a part of the natural response to pathogens (see Fehr, T., *et al.*, *J Exp. Med.* 185:1785-1792 (1997)). Thus, antibodies presented by a highly organized viral surface are able to induce strong anti-antibody responses.

30 As indicated, however, the immune system usually fails to produce antibodies against self-derived structures. For soluble antigens present at low concentrations, this is due to tolerance at the Th cell level. Under these conditions, coupling the self-

antigen to a carrier that can deliver T help may break tolerance. For soluble proteins present at high concentrations or membrane proteins at low concentration, B and Th cells may be tolerant. However, B cell tolerance may be reversible (anergy) and can be broken by administration of the antigen in a highly organized fashion coupled to a foreign carrier (Bachmann and Zinkernagel, *Ann. Rev. Immunol.* 15:235-270 (1997)). As shown in pending U.S. Application No. 10/050,902 filed on January 18, 2002, strong immune responses could be induced with compositions comprising A β peptides (A β 1-15, A β 1-27 and A β 33-42, which is a self-antigen in mice) bound to a VLP. In particular, the abovementioned human A β peptides bound to the VLP of RNA phage Q β induced high A β specific titers in human APP transgenic mice (described in Example) demonstrating that tolerance to the self-antigen A β could be overcome by immunizing with A β peptides bound to a VLP.

There is thus a need for highly immunogenic safe compositions and vaccines, respectively, to treat Alzheimer diseases, in particular, using immunogens devoid of T-cell epitopes and adjuvants, respectively, which might elicit side-effects, and still being capable of inducing high antibody titers, which antibodies, furthermore, being capable of binding to amyloid plaques.

BRIEF SUMMARY OF THE INVENTION

We have now found that A β 1-6 peptide, which is bound to a core particle having a structure with an inherent repetitive organization, and hereby in particular to virus-like-particles (VLPs) and subunits of VLPs, respectively, leading to highly ordered and repetitive conjugates represent a potent immunogen for the induction of antibodies specific for A β 1-6. Therefore, the present invention provides a prophylactic and therapeutic mean for the treatment of Alzheimer's disease, which is based on an ordered and repetitive A β 1-6 -core particle array, and in particular on a VLP-A β 1-6 peptide conjugate and -array, respectively. This prophylactic and therapeutic is able to induce high titers of anti-A β 1-6 peptide antibodies, which are cross-reactive to soluble A β and are capable of binding to human amyloid plaques of a human APP transgenic mouse model and to AD amyloid plaques. Furthermore, the elicited antibodies do not bind to APP on brain sections.

Moreover, the present invention provides for new compositions, vaccines and methods of treatment of AD. The compositions and vaccines comprising A β 1-6 peptides are devoid of T-cell epitopes and induce antibodies binding AD plaques and soluble A β . The A β 1-6 peptides are presented to the immune system of the patient
5 in a highly repetitive and ordered fashion through binding of the A β peptides or to a core particle, preferably to a VLP, and even more preferably to a VLP of a RNA phage.

In a preferred embodiment, the antigen or antigenic determinant is the human amyloid beta peptide A β 1-6 (DAEFRH; SEQ ID NO: 75) being a fragment of A β
10 (DAEFRHDSGYEVHHQKLVFFAEDVGSNKGAIIGLMVGGVVIA (SEQ ID NO: 91), wherein the human amyloid beta peptide A β 1-6 is bound to the core particle and VLP, respectively. The amyloid beta protein is provided in SEQ ID NO: 92. The amyloid beta precursor protein is provided in SEQ ID NO: 93.

The present invention, thus, provides for a composition comprising: (a) a core
15 particle with at least one first attachment site; and (b) at least one antigen or antigenic determinant with at least one second attachment site, wherein said antigen or antigenic determinant is a A β 1-6 peptide, and wherein said second attachment site being selected from the group consisting of (i) an attachment site not naturally occurring with said antigen or antigenic determinant; and (ii) an attachment site
20 naturally occurring with said antigen or antigenic determinant, wherein said second attachment site is capable of association to said first attachment site; and wherein said antigen or antigenic determinant and said core particle interact through said association to form an ordered and repetitive antigen array. Preferred embodiments of core particles suitable for use in the present invention are a virus, a virus-like
25 particle, a bacteriophage, a virus-like particle of a RNA-phage, a bacterial pilus or flagella or any other core particle having an inherent repetitive structure capable of forming an ordered and repetitive antigen array in accordance with the present invention.

The A β fragments of the present invention are soluble and generally do not
30 form aggregates. Moreover, they are bound, and preferably covalently bound to a core particle and VLP, respectively. Therefore, the compositions of the invention do not bear the risk of inducing toxic effects such as seeding of amyloid deposition.

It is an unexpected finding of this invention that a high titer of antibodies cross-reactive with soluble A β and AD amyloid plaques could be obtained with a composition comprising the A β 1-6 peptide bound to a core particle and VLP, respectively. In particular, VLP have been shown to mediate induction of antibodies
5 against self antigens, thus breaking self-tolerance (WO 02/056905, the disclosure of which is herewith incorporated by reference in its entirety). Furthermore, the small size of this epitope precludes the presence of T-cell epitopes, thus providing new compositions that do not induce A β specific T-cell responses. In addition, the elicited antibodies do not bind to APP on brain sections. Thus, the present invention provides
10 for a safe vaccine composition for the prevention and treatment of AD.

More specifically, the invention provides a composition comprising an ordered and repetitive antigen or antigenic determinant array, and hereby in particular A β 1-6 peptide VLP conjugates. More specifically, the invention provides a composition comprising a virus-like particle and at least one A β 1-6 peptide bound thereto. The
15 invention also provides a process for producing the conjugates and the ordered and repetitive arrays, respectively. The compositions of the invention are useful in the production of vaccines for the treatment of Alzheimer's disease and as a pharmaccine to prevent or cure Alzheimer's disease and to efficiently induce immune responses, in particular antibody responses. Furthermore, the compositions
20 of the invention are particularly useful to efficiently induce self-specific immune responses within the indicated context.

In the present invention, a A β 1-6 peptide is bound to a core particle and VLP, respectively, typically in an oriented manner, yielding an ordered and repetitive A β 1-6 peptide antigen array. Furthermore, the highly repetitive and organized structure of
25 the core particles and VLPs, respectively, mediates the display of the A β peptide in a highly ordered and repetitive fashion leading to a highly organized and repetitive antigen array. Furthermore, binding of the A β 1-6 peptide to the core particle and VLP, respectively, provides T helper cell epitopes, since the core particle and VLP is foreign to the host immunized with the core particle- A β 1-6 peptide array and VLP-
30 A β 1-6 peptide array, respectively. Those arrays differ from prior art conjugates, in particular, in their highly organized structure, dimensions, and in the repetitiveness of the antigen on the surface of the array.

In one aspect of the invention, the A β 1-6 peptide is chemically synthesized, while the core particle and the VLP, respectively, is expressed and purified from an expression host suitable for the folding and assembly of the core particle and the VLP, respectively. The A β 1-6 peptide array is then assembled by binding the A β 1-6 peptide to the core particle and the VLP, respectively.

In another aspect, the present invention provides for a composition comprising (a) a virus-like particle, and (b) at least one antigen or antigenic determinant, wherein said antigen or said antigenic determinant is an A β 1-6 peptide, and wherein said at least one antigen or antigenic determinant is bound to said virus-like particle.

In a further aspect, the present invention provides for a pharmaceutical composition comprising (a) the inventive composition, and (b) an acceptable pharmaceutical carrier.

In still a further aspect, the present invention provides for a vaccine composition comprising a composition, wherein said composition comprising (a) a virus-like particle; and (b) at least one antigen or antigenic determinant, wherein said antigen or said antigenic determinant is a A β 1-6 peptide; and wherein said at least one antigen or antigenic determinant is bound to said virus-like particle.

In another aspect, the present invention provides for a method of immunization comprising administering the inventive composition, the inventive pharmaceutical composition or the inventive vaccine to an animal.

In still a further aspect, the present invention provides for a process for producing an inventive composition comprising (a) providing a virus-like particle; and (b) providing at least one antigen or antigenic determinant, wherein said antigen or said antigenic determinant is a A β 1-6 peptide; (c) combining said virus-like particle and said at least one antigen or antigenic determinant so that said at least one antigen or antigenic determinant is bound to said virus-like particle.

Analogously, the present invention provides a process for producing a composition of claim 1 comprising: (a) providing a core particle with at least one first attachment site; (b) providing at least one antigen or antigenic determinant with at least one second attachment site, wherein said antigen or antigenic determinant is a A β 1-6 peptide, and wherein said second attachment site being selected from the group consisting of (i) an attachment site not naturally occurring with said antigen or

antigenic determinant; and (ii) an attachment site naturally occurring with said antigen or antigenic determinant; and wherein said second attachment site is capable of association to said first attachment site; and (c) combining said core particle and said at least one antigen or antigenic determinant, wherein said antigen or antigenic determinant and said core particle interact through said association to form an ordered and repetitive antigen array.

In a further aspect, the present invention provides for a use of a composition of claim 1 for the manufacture of a medicament for treatment of Alzheimer's disease.

In a still further aspect, the present invention provides for a use of a composition of claim 1 for the preparation of a medicament for the therapeutic or prophylactic treatment of Alzheimer's disease. Furthermore, in a still further aspect, the present invention provides for a use of a composition of claim 1, either in isolation or in combination with other agents, or with explicit absence of specific substances such as adjuvants, for the manufacture of a composition, pharmaceutical composition, vaccine, drug or medicament for therapy or prophylaxis of Alzheimer's disease, and/or for stimulating the mammalian immune system.

Therefore, the invention provides, in particular, vaccine compositions which are suitable for preventing and/or attenuating Alzheimer's disease or conditions related thereto. The invention further provides immunization and vaccination methods, respectively, for preventing and/or attenuating Alzheimer's disease or conditions related thereto in humans. The inventive compositions may be used prophylactically or therapeutically.

In specific embodiments, the invention provides methods for preventing and/or attenuating Alzheimer's disease or conditions related thereto which are caused or exacerbated by "self" gene products, i.e. "self antigens" as used herein. In related embodiments, the invention provides methods for inducing immunological responses in animals and individuals, respectively, which lead to the production of antibodies that prevent and/or attenuate Alzheimer's disease or conditions related thereto, which are caused or exacerbated by "self" gene products.

As would be understood by one of ordinary skill in the art, when compositions of the invention are administered to an animal or a human, they may be in a composition which contains salts, buffers, adjuvants, or other substances which are

desirable for improving the efficacy of the composition. Examples of materials suitable for use in preparing pharmaceutical compositions are provided in numerous sources including *Remington's Pharmaceutical Sciences* (Osol, A, ed., Mack Publishing Co. (1990)).

5 Compositions of the invention are said to be "pharmacologically acceptable" if their administration can be tolerated by a recipient individual. Further, the compositions of the invention will be administered in a "therapeutically effective amount" (*i.e.*, an amount that produces a desired physiological effect).

10 The compositions of the present invention may be administered by various methods known in the art, but will normally be administered by injection, infusion, inhalation, oral administration, or other suitable physical methods. The compositions may alternatively be administered intramuscularly, intravenously, or subcutaneously. Components of compositions for administration include sterile aqueous (*e.g.*, physiological saline) or non-aqueous solutions and suspensions. Examples of non-
15 aqueous solvents are propylene glycol, polyethylene glycol, vegetable oils such as olive oil, and injectable organic esters such as ethyl oleate. Carriers or occlusive dressings can be used to increase skin permeability and enhance antigen absorption.

20 Other embodiments of the present invention will be apparent to one of ordinary skill in light of what is known in the art, the following drawings and description of the invention, and the claims.

BRIEF DESCRIPTION OF THE FIGURES

FIG. 1 depicts the SDS-PAGE gel, run under reducing conditions, showing the result of the coupling of the A β 1-6 peptide (NH₂-DAEFRHGGC-CONH₂) (SEQ ID NO: 77) to the VLP of Q β coat protein.

25 FIG. 2 shows the ELISA analysis of the antibodies specific for A β 1-6 in sera of mice immunized with A β 1-6 peptide coupled to the VLP of Q β coat protein.

FIG. 3 shows the ELISA analysis of the antibodies specific for A β 1-40 in sera of mice immunized with A β 1-6 peptide coupled to the VLP of Q β coat protein.

FIG. 4 A-B show a brain section of an APP23 mouse (A) and an entorhinal cortex section from an AD patient (B) stained with sera of mice immunized with A β 1-6 peptide coupled to the VLP of Q β coat protein.

FIG. 5 A-E show brain sections of an APP23 mouse stained with sera of mice
5 immunized with A β 1-6 peptide coupled to the VLP of Q β coat protein, or with a polyclonal rabbit antiserum specific for the C-terminus of human or mouse APP.

FIG.6 shows the result of the immunization of rhesus monkeys with human A β 1-6 coupled to Q β VLP as measured in an ELISA assay.

FIG. 7 shows the result of the binding to plaques of sera from monkeys
10 immunized with human A β 1-6 coupled to Q β VLP, as measured by histology on human AD and transgenic mouse plaques.

FIG. 8 depicts the SDS-PAGE analysis of the coupling of murine A β 1-6 to AP205 VLP.

FIG. 9 shows the result of the immunisation of mice with murine A β 1-6
15 coupled to AP205 as measured in an ELISA assay.

FIG. 10 shows the analysis by ELISA of the anti-A β 40 and anti-A β 42 titers in the sera of "Swedish/London" transgenic mice immunized with Q β hA β 1-6 between 9.5 and 19 months of age.

FIG. 11 shows the immunohistochemical staining of brain sections of
20 "Swedish/London" transgenic mice immunized with Q β hA β 1-6 or PBS .

FIG. 12 shows the quantification of plaque deposition in "Swedish/London" transgenic mice immunized with Q β hA β 1-6, Q β or PBS between 9.5 and 19 months of age.

FIG. 13 shows the quantification of plaque deposition in "Swedish/London"
25 transgenic mice immunized with Q β hA β 1-6 or PBS between 13.5 and 19 months of age.

FIG. 14 shows the analysis by ELISA of the anti-A β 40 and anti-A β 42 titers in the sera of "Swedish" transgenic mice immunized with Q β hA β 1-6.

FIG. 15 shows the immunohistochemical staining of brain sections from
30 "Swedish" transgenic mice immunized with Q β hA β 1-6 or PBS.

FIG. 16 shows the quantification of plaque deposition in "Swedish" transgenic mice immunized with Q β hA β 1-6 or PBS.

DETAILED DESCRIPTION OF THE INVENTION

Unless defined otherwise, all technical and scientific terms used herein have the same meanings as commonly understood by one of ordinary skill in the art to which this invention belongs. Although any methods and materials similar or equivalent to those described herein can be used in the practice or testing of the present invention, the preferred methods and materials are hereinafter described.

1. Definitions:

A β 1-6 peptide: An A β 1-6 peptide as used herein refers to peptides having a sequence corresponding to the human A β 1-6 sequence, or homologous to the human A β 1-6 sequence. Sequences homologous to the human A β 1-6 sequence include, but are not limited to the A β 1-6 sequences of other species and hereby including, but not limited to, the sequence of primate, rabbit, guinea pig, *Xenopus Laevis*, frog, mouse and rat A β 1-6. The A β 1-6 sequences from *Xenopus Laevis* or frog, although differing from human A β 1-6 at two positions, have conservative mutations (Ala-Ser, Phe-Tyr), and are still considered to be homologous to A β 1-6 in accordance with this definition. In accordance with the present invention, however, the A β 1-6 peptide is typically modified, such that a second attachment site is attached thereto. Preferably, the second attachment site is modified with a linker or an amino acid linker comprising a second attachment site for binding to a core particle and VLP, respectively. While referring herein to A β 1-6 peptides, a modified A β 1-6 peptide, as indicated above, i.e. A β 1-6 peptides with a second attachment site attached thereto, shall be encompassed. Typically, however, the modifications are explicitly indicated in the specification. Further preferred embodiments of an A β 1-6 peptide being an antigen or antigenic determinant in accordance with the present invention become apparent as this specification proceeds.

Adjuvant: The term "adjuvant" as used herein refers to non-specific stimulators of the immune response or substances that allow generation of a depot in the host

which when combined with the vaccine and pharmaceutical composition, respectively, of the present invention may provide for an even more enhanced immune response. A variety of adjuvants can be used. Examples include complete and incomplete Freund's adjuvant, aluminum hydroxide and modified
5 muramyldipeptide. Further adjuvants are mineral gels such as aluminum hydroxide, surface active substances such as lysolecithin, pluronic polyols, polyanions, peptides, oil emulsions, keyhole limpet hemocyanins, dinitrophenol, and potentially useful human adjuvants such as BCG (bacille Calmette-Guerin) and *Corynebacterium parvum*. Such adjuvants are also well known in the art. Further adjuvants that can
10 be administered with the compositions of the invention include, but are not limited to, Monophosphoryl lipid immunomodulator, AdjuVax 100a, QS-21, QS-18, CRL1005, Aluminum salts (Alum), MF-59, OM-174, OM-197, OM-294, and Virosomal adjuvant technology. The adjuvants can also comprise a mixture of these substances.

15 Immunologically active saponin fractions having adjuvant activity derived from the bark of the South American tree Quillaja Saponaria Molina are known in the art. For example QS21, also known as QA21, is an Hplc purified fraction from the Quillaja Saponaria Molina tree and it's method of its production is disclosed (as QA21) in U.S. Pat. No. 5,057,540. Quillaja saponin has also been disclosed as an
20 adjuvant by Scott et al, Int. Archs. Allergy Appl. Immun., 1985, 77, 409. Monosphoryl lipid A and derivatives thereof are known in the art. A preferred derivative is 3 de-o-acylated monophosphoryl lipid A, and is known from British Patent No. 2220211. Further preferred adjuvants are described in WO00/00462, the disclosure of which is herein incorporated by reference.

25 However, an advantageous feature of the present invention is the high immunogenicity of the inventive compositions. As already outlined herein or will become apparent as this specification proceeds, vaccines and pharmaceutical compositions devoid of adjuvants are provided, in further alternative or preferred embodiments, leading to vaccines and pharmaceutical compositions for treating AD
30 being devoid of adjuvants and, thus, having a superior safety profile since adjuvants may cause side-effects. The term "devoid" as used herein in the context of vaccines

and pharmaceutical compositions for treating AD refers to vaccines and pharmaceutical compositions that are used without adjuvants.

Amino acid linker: An "amino acid linker", or also just termed "linker" within this specification, as used herein, either associates the antigen or antigenic determinant with the second attachment site, or more preferably, already comprises or contains the second attachment site, typically - but not necessarily - as one amino acid residue, preferably as a cysteine residue. The term "amino acid linker" as used herein, however, does not intend to imply that such an amino acid linker consists exclusively of amino acid residues, even if an amino acid linker consisting of amino acid residues is a preferred embodiment of the present invention. The amino acid residues of the amino acid linker are, preferably, composed of naturally occurring amino acids or unnatural amino acids known in the art, all-L or all-D or mixtures thereof. However, an amino acid linker comprising a molecule with a sulfhydryl group or cysteine residue is also encompassed within the invention. Such a molecule comprise preferably a C1-C6 alkyl-, cycloalkyl (C5,C6), aryl or heteroaryl moiety. However, in addition to an amino acid linker, a linker comprising preferably a C1-C6 alkyl-, cycloalkyl- (C5,C6), aryl- or heteroaryl- moiety and devoid of any amino acid(s) shall also be encompassed within the scope of the invention. Association between the antigen or antigenic determinant or optionally the second attachment site and the amino acid linker is preferably by way of at least one covalent bond, more preferably by way of at least one peptide bond.

Animal: As used herein, the term "animal" is meant to include, for example, humans, sheep, elks, deer, mule deer, minks, mammals, monkeys, horses, cattle, pigs, goats, dogs, cats, rats, mice, birds, chicken, reptiles, fish, insects and arachnids.

Antibody: As used herein, the term "antibody" refers to molecules which are capable of binding an epitope or antigenic determinant. The term is meant to include whole antibodies and antigen-binding fragments thereof, including single-chain antibodies. Most preferably the antibodies are human antigen binding antibody fragments and include, but are not limited to, Fab, Fab' and F(ab')₂, Fd, single-chain Fvs (scFv), single-chain antibodies, disulfide-linked Fvs (sdFv) and fragments comprising either a V_L or V_H domain. The antibodies can be from any animal origin including birds and mammals. Preferably, the antibodies are human, murine, rabbit,

goat, guinea pig, camel, horse or chicken. As used herein, "human" antibodies include antibodies having the amino acid sequence of a human immunoglobulin and include antibodies isolated from human immunoglobulin libraries or from animals transgenic for one or more human immunoglobulins and that do not express
5 endogenous immunoglobulins, as described, for example, in U.S. Patent No. 5,939,598 by Kucherlapati *et al.*

Antigen: As used herein, the term "antigen" refers to a molecule capable of being bound by an antibody or a T cell receptor (TCR) if presented by MHC molecules. The term "antigen", as used herein, also encompasses T-cell epitopes.
10 An antigen is additionally capable of being recognized by the immune system and/or being capable of inducing a humoral immune response and/or cellular immune response leading to the activation of B- and/or T-lymphocytes. This may, however, require that, at least in certain cases, the antigen contains or is linked to a Th cell epitope and is given in adjuvant. An antigen can have one or more epitopes (B- and
15 T- epitopes). The specific reaction referred to above is meant to indicate that the antigen will preferably react, typically in a highly selective manner, with its corresponding antibody or TCR and not with the multitude of other antibodies or TCRs which may be evoked by other antigens. Antigens as used herein may also be mixtures of several individual antigens.

20 Antigenic determinant: As used herein, the term "antigenic determinant" is meant to refer to that portion of an antigen that is specifically recognized by either B- or T-lymphocytes. B-lymphocytes responding to antigenic determinants produce antibodies, whereas T-lymphocytes respond to antigenic determinants by proliferation and establishment of effector functions critical for the mediation of
25 cellular and/or humoral immunity.

Association: As used herein, the term "association" as it applies to the first and second attachment sites, refers to the binding of the first and second attachment sites that is preferably by way of at least one non-peptide bond. The nature of the association may be covalent, ionic, hydrophobic, polar or any combination thereof,
30 preferably the nature of the association is covalent.

Attachment Site, First: As used herein, the phrase "first attachment site" refers to an element of non-natural or natural origin, to which the second attachment site

located on the antigen or antigenic determinant may associate. The first attachment site may be a protein, a polypeptide, an amino acid, a peptide, a sugar, a polynucleotide, a natural or synthetic polymer, a secondary metabolite or compound (biotin, fluorescein, retinol, digoxigenin, metal ions, phenylmethylsulfonylfluoride),
5 or a combination thereof, or a chemically reactive group thereof. The first attachment site is located, typically and preferably on the surface, of the core particle such as, preferably the virus-like particle. Multiple first attachment sites are present on the surface of the core and virus-like particle, respectively, typically in a repetitive configuration.

10 Attachment Site, Second: As used herein, the phrase "second attachment site" refers to an element associated with the antigen or antigenic determinant to which the first attachment site located on the surface of the core particle and virus-like particle, respectively, may associate. The second attachment site of the antigen or antigenic determinant may be a protein, a polypeptide, a peptide, a sugar, a polynucleotide, a
15 natural or synthetic polymer, a secondary metabolite or compound (biotin, fluorescein, retinol, digoxigenin, metal ions, phenylmethylsulfonylfluoride), or a combination thereof, or a chemically reactive group thereof. At least one second attachment site is present on the antigen or antigenic determinant. The term "antigen or antigenic determinant with at least one second attachment site" refers, therefore, to
20 an antigen or antigenic construct comprising at least the antigen or antigenic determinant and the second attachment site. However, in particular for a second attachment site, which is of non-natural origin, i.e. not naturally occurring within the antigen or antigenic determinant, these antigen or antigenic constructs comprise an "amino acid linker".

25 Bound: As used herein, the term "bound" refers to binding or attachment that may be covalent, *e.g.*, by chemically coupling, or non-covalent, *e.g.*, ionic interactions, hydrophobic interactions, hydrogen bonds, etc. Covalent bonds can be, for example, ester, ether, phosphoester, amide, peptide, imide, carbon-sulfur bonds, carbon-phosphorus bonds, and the like. The term "bound" is broader than and
30 includes terms such as "coupled," "fused" and "attached".

Coat protein(s): As used herein, the term "coat protein(s)" refers to the protein(s) of a bacteriophage or a RNA-phage capable of being incorporated within

the capsid assembly of the bacteriophage or the RNA-phage. However, when referring to the specific gene product of the coat protein gene of RNA-phages the term "CP" is used. For example, the specific gene product of the coat protein gene of RNA-phage Q β is referred to as "Q β CP", whereas the "coat proteins" of bacteriophage Q β comprise the "Q β CP" as well as the A1 protein. The capsid of Bacteriophage Q β is composed mainly of the Q β CP, with a minor content of the A1 protein. Likewise, the VLP Q β coat protein contains mainly Q β CP, with a minor content of A1 protein.

Core particle: As used herein, the term "core particle" refers to a rigid structure with an inherent repetitive organization. A core particle as used herein may be the product of a synthetic process or the product of a biological process.

Coupled: The term "coupled", as used herein, refers to attachment by covalent bonds or by strong non-covalent interactions, typically and preferably to attachment by covalent bonds. Any method normally used by those skilled in the art for the coupling of biologically active materials can be used in the present invention.

Effective Amount: As used herein, the term "effective amount" refers to an amount necessary or sufficient to realize a desired biologic effect. An effective amount of the composition would be the amount that achieves this selected result, and such an amount could be determined as a matter of routine by a person skilled in the art. For example, an effective amount for treating an immune system deficiency could be that amount necessary to cause activation of the immune system, resulting in the development of an antigen specific immune response upon exposure to antigen. The term is also synonymous with "sufficient amount."

The effective amount for any particular application can vary depending on such factors as the disease or condition being treated, the particular composition being administered, the size of the subject, and/or the severity of the disease or condition. One of ordinary skill in the art can empirically determine the effective amount of a particular composition of the present invention without necessitating undue experimentation.

Epitope: As used herein, the term "epitope" refers to continuous or discontinuous portions of a polypeptide having antigenic or immunogenic activity in an animal, preferably a mammal, and most preferably in a human. An epitope is

recognized by an antibody or a T cell through its T cell receptor in the context of an MHC molecule. An "immunogenic epitope," as used herein, is defined as a portion of a polypeptide that elicits an antibody response or induces a T-cell response in an animal, as determined by any method known in the art. (See, for example, Geysen *et al.*, *Proc. Natl. Acad. Sci. USA* 81:3998-4002 (1983)). The term "antigenic epitope," as used herein, is defined as a portion of a protein to which an antibody can immunospecifically bind its antigen as determined by any method well known in the art. Immunospecific binding excludes non-specific binding but does not necessarily exclude cross-reactivity with other antigens. Antigenic epitopes need not necessarily be immunogenic. Antigenic epitopes can also be T-cell epitopes, in which case they can be bound immunospecifically by a T-cell receptor within the context of an MHC molecule.

An epitope can comprise 3 amino acids in a spatial conformation which is unique to the epitope. Generally, an epitope consists of at least about 5 such amino acids, and more usually, consists of at least about 8-10 such amino acids. If the epitope is an organic molecule, it may be as small as Nitrophenyl.

Fusion: As used herein, the term "fusion" refers to the combination of amino acid sequences of different origin in one polypeptide chain by in-frame combination of their coding nucleotide sequences. The term "fusion" explicitly encompasses internal fusions, *i.e.*, insertion of sequences of different origin within a polypeptide chain, in addition to fusion to one of its termini.

Immune response: As used herein, the term "immune response" refers to a humoral immune response and/or cellular immune response leading to the activation or proliferation of B- and/or T-lymphocytes and/or and antigen presenting cells. In some instances, however, the immune responses may be of low intensity and become detectable only when using at least one substance in accordance with the invention. "Immunogenic" refers to an agent used to stimulate the immune system of a living organism, so that one or more functions of the immune system are increased and directed towards the immunogenic agent. An "immunogenic polypeptide" is a polypeptide that elicits a cellular and/or humoral immune response, whether alone or linked to a carrier in the presence or absence of an adjuvant. Preferably, antigen presenting cell may be activated.

A substance which "enhances" an immune response refers to a substance in which an immune response is observed that is greater or intensified or deviated in any way with the addition of the substance when compared to the same immune response measured without the addition of the substance. For example, the lytic activity of cytotoxic T cells can be measured, *e.g.* using a ^{51}Cr release assay, in samples obtained with and without the use of the substance during immunization. The amount of the substance at which the CTL lytic activity is enhanced as compared to the CTL lytic activity without the substance is said to be an amount sufficient to enhance the immune response of the animal to the antigen. In a preferred embodiment, the immune response is enhanced by a factor of at least about 2, more preferably by a factor of about 3 or more. The amount or type of cytokines secreted may also be altered. Alternatively, the amount of antibodies induced or their subclasses may be altered.

Immunization: As used herein, the terms "immunize" or "immunization" or related terms refer to conferring the ability to mount a substantial immune response (comprising antibodies and/or cellular immunity such as effector CTL) against a target antigen or epitope. These terms do not require that complete immunity be created, but rather that an immune response be produced which is substantially greater than baseline. For example, a mammal may be considered to be immunized against a target antigen if the cellular and/or humoral immune response to the target antigen occurs following the application of methods of the invention.

Natural origin: As used herein, the term "natural origin" means that the whole or parts thereof are not synthetic and exist or are produced in nature.

Non-natural: As used herein, the term generally means not from nature, more specifically, the term means from the hand of man.

Non-natural origin: As used herein, the term "non-natural origin" generally means synthetic or not from nature; more specifically, the term means from the hand of man.

Ordered and repetitive antigen or antigenic determinant array: As used herein, the term "ordered and repetitive antigen or antigenic determinant array" generally refers to a repeating pattern of antigen or antigenic determinant, characterized by a typically and preferably uniform spacial arrangement of the antigens or antigenic

determinants with respect to the core particle and virus-like particle, respectively. In one embodiment of the invention, the repeating pattern may be a geometric pattern. Typical and preferred examples of suitable ordered and repetitive antigen or antigenic determinant arrays are those which possess strictly repetitive
5 paracrystalline orders of antigens or antigenic determinants, preferably with spacings of 0.5 to 30 nanometers, more preferably 5 to 15 nanometers.

Pili: As used herein, the term "pili" (singular being "pilus") refers to extracellular structures of bacterial cells composed of protein monomers (*e.g.*, pilin monomers) which are organized into ordered and repetitive patterns. Further, pili are
10 structures which are involved in processes such as the attachment of bacterial cells to host cell surface receptors, inter-cellular genetic exchanges, and cell-cell recognition. Examples of pili include Type-1 pili, P-pili, F1C pili, S-pili, and 987P-pili. Additional examples of pili are set out below.

Pilus-like structure: As used herein, the phrase "pilus-like structure" refers to
15 structures having characteristics similar to that of pili and composed of protein monomers. One example of a "pilus-like structure" is a structure formed by a bacterial cell which expresses modified pilin proteins that do not form ordered and repetitive arrays that are identical to those of natural pili.

Polypeptide: As used herein, the term "polypeptide" refers to a molecule
20 composed of monomers (amino acids) linearly linked by amide bonds (also known as peptide bonds). It indicates a molecular chain of amino acids and does not refer to a specific length of the product. Thus, peptides, dipeptides, tripeptides, oligopeptides and proteins are included within the definition of polypeptide. This term is also intended to refer to post-expression modifications of the polypeptide, for example,
25 glycosolations, acetylations, phosphorylations, and the like. A recombinant or derived polypeptide is not necessarily translated from a designated nucleic acid sequence. It may also be generated in any manner, including chemical synthesis.

Residue: As used herein, the term "residue" is meant to mean a specific amino acid in a polypeptide backbone or side chain.

30 Self antigen: As used herein, the tem "self antigen" refers to proteins encoded by the host's DNA and products generated by proteins or RNA encoded by the host's DNA are defined as self. In addition, proteins that result from a combination of two

or several self-molecules or that represent a fraction of a self-molecule and proteins that have a high homology two self-molecules as defined above (>95%, preferably >97%, more preferably >99%) may also be considered self.

Treatment: As used herein, the terms "treatment", "treat", "treated" or
5 "treating" refer to prophylaxis and/or therapy. When used with respect to an infectious disease, for example, the term refers to a prophylactic treatment which increases the resistance of a subject to infection with a pathogen or, in other words, decreases the likelihood that the subject will become infected with the pathogen or will show signs of illness attributable to the infection, as well as a treatment after the
10 subject has become infected in order to fight the infection, *e.g.*, reduce or eliminate the infection or prevent it from becoming worse.

Vaccine: As used herein, the term "vaccine" refers to a formulation which contains the composition of the present invention and which is in a form that is capable of being administered to an animal. Typically, the vaccine comprises a
15 conventional saline or buffered aqueous solution medium in which the composition of the present invention is suspended or dissolved. In this form, the composition of the present invention can be used conveniently to prevent, ameliorate, or otherwise treat a condition. Upon introduction into a host, the vaccine is able to provoke an immune response including, but not limited to, the production of antibodies and/or
20 cytokines and/or the activation of cytotoxic T cells, antigen presenting cells, helper T cells, dendritic cells and/or other cellular responses.

Optionally, the vaccine of the present invention additionally includes an adjuvant which can be present in either a minor or major proportion relative to the compound of the present invention.

25 Virus-like particle (VLP): As used herein, the term "virus-like particle" refers to a structure resembling a virus particle. Moreover, a virus-like particle in accordance with the invention is non replicative and noninfectious since it lacks all or part of the viral genome, in particular the replicative and infectious components of the viral genome. A virus-like particle in accordance with the invention may contain
30 nucleic acid distinct from their genome. A typical and preferred embodiment of a virus-like particle in accordance with the present invention is a viral capsid such as the viral capsid of the corresponding virus, bacteriophage, or RNA-phage. The terms

“viral capsid” or “capsid”, as interchangeably used herein, refer to a macromolecular assembly composed of viral protein subunits. Typically and preferably, the viral protein subunits assemble into a viral capsid and capsid, respectively, having a structure with an inherent repetitive organization, wherein said structure is, typically, spherical or tubular. For example, the capsids of RNA-phages or HBcAg’s have a spherical form of icosahedral symmetry. The term “capsid-like structure” as used herein, refers to a macromolecular assembly composed of viral protein subunits resembling the capsid morphology in the above defined sense but deviating from the typical symmetrical assembly while maintaining a sufficient degree of order and repetitiveness.

Virus-like particle of a bacteriophage: As used herein, the term "virus-like particle of a bacteriophage" refers to a virus-like particle resembling the structure of a bacteriophage, being non replicative and noninfectious, and lacking at least the gene or genes encoding for the replication machinery of the bacteriophage, and typically also lacking the gene or genes encoding the protein or proteins responsible for viral attachment to or entry into the host. This definition should, however, also encompass virus-like particles of bacteriophages, in which the aforementioned gene or genes are still present but inactive, and, therefore, also leading to non-replicative and noninfectious virus-like particles of a bacteriophage.

VLP of RNA phage coat protein: The capsid structure formed from the self-assembly of 180 subunits of RNA phage coat protein and optionally containing host RNA is referred to as a “VLP of RNA phage coat protein”. A specific example is the VLP of Q β coat protein. In this particular case, the VLP of Q β coat protein may either be assembled exclusively from Q β CP subunits (generated by expression of a Q β CP gene containing, for example, a TAA stop codon precluding any expression of the longer A1 protein through suppression, see Kozlovskaya, T.M., *et al.*, *Intervirology* 39: 9-15 (1996)), or additionally contain A1 protein subunits in the capsid assembly.

Virus particle: The term "virus particle" as used herein refers to the morphological form of a virus. In some virus types it comprises a genome surrounded by a protein capsid; others have additional structures (*e.g.*, envelopes, tails, etc.).

One, a, or an: When the terms "one," "a," or "an" are used in this disclosure, they mean "at least one" or "one or more," unless otherwise indicated.

As will be clear to those skilled in the art, certain embodiments of the invention involve the use of recombinant nucleic acid technologies such as cloning,
5 polymerase chain reaction, the purification of DNA and RNA, the expression of recombinant proteins in prokaryotic and eukaryotic cells, etc. Such methodologies are well known to those skilled in the art and can be conveniently found in published laboratory methods manuals (*e.g.*, Sambrook, J. *et al.*, eds., *Molecular Cloning, A Laboratory Manual*, 2nd. edition, Cold Spring Harbor Laboratory Press, Cold Spring
10 Harbor, N.Y. (1989); Ausubel, F. *et al.*, eds., *Current Protocols in Molecular Biology*, John H. Wiley & Sons, Inc. (1997)). Fundamental laboratory techniques for working with tissue culture cell lines (Celis, J., ed., *Cell Biology*, Academic Press, 2nd edition, (1998)) and antibody-based technologies (Harlow, E. and Lane, D., *Antibodies: A Laboratory Manual*, Cold Spring Harbor Laboratory, Cold Spring
15 Harbor, N.Y. (1988); Deutscher, M.P., "Guide to Protein Purification," *Meth. Enzymol.* 128, Academic Press San Diego (1990); Scopes, R.K., *Protein Purification Principles and Practice*, 3rd ed., Springer-Verlag, New York (1994)) are also adequately described in the literature, all of which are incorporated herein by reference.

20 2. Compositions and Methods for Enhancing an Immune Response

The disclosed invention provides compositions and methods for inducing an immune response against A β 1-6 peptide in an animal, inducing antibodies capable of binding A β amyloid plaques and soluble A β . Compositions of the invention comprise, or alternatively consist of (a) a core particle with at least one first
25 attachment site; and (b) at least one antigen or antigenic determinant with at least one second attachment site, wherein said antigen or antigenic determinant is an A β 1-6 peptide, and wherein said second attachment site being selected from the group consisting of (i) an attachment site not naturally occurring with said antigen or antigenic determinant; and (ii) an attachment site naturally occurring with said
30 antigen or antigenic determinant, wherein said second attachment site is capable of association to said first attachment site; and wherein said antigen or antigenic

determinant and said core particle interact through said association to form an ordered and repetitive antigen array. More specifically, compositions of the invention comprise, or alternatively consist of, a virus-like particle and at least one antigen or antigenic determinant, wherein the antigen or antigenic determinant is a A β 1-6 peptide, and wherein the at least one antigen or antigenic determinant is bound to the virus-like particle so as to form an ordered and repetitive antigen-VLP-array. Furthermore, the invention conveniently enables the practitioner to construct such a composition, inter alia, for treatment and/or prophylactic prevention of Alzheimer's disease. Virus-like particles in the context of the present application refer to structures resembling a virus particle but which are not pathogenic. In general, virus-like particles lack the viral genome and, therefore, are noninfectious. Also, virus-like particles can be produced in large quantities by heterologous expression and can be easily purified.

In one embodiment, the core particle comprises, or is selected from a group consisting of, a virus, a bacterial pilus, a structure formed from bacterial pilin, a bacteriophage, a virus-like particle, a virus-like particle of a RNA phage, a viral capsid particle or a recombinant form thereof. Any virus known in the art having an ordered and repetitive coat and/or core protein structure may be selected as a core particle of the invention; examples of suitable viruses include sindbis and other alphaviruses, rhabdoviruses (*e.g.* vesicular stomatitis virus), picornaviruses (*e.g.*, human rhino virus, Aichi virus), togaviruses (*e.g.*, rubella virus), orthomyxoviruses (*e.g.*, Thogoto virus, Batken virus, fowl plague virus), polyomaviruses (*e.g.*, polyomavirus BK, polyomavirus JC, avian polyomavirus BFDV), parvoviruses, rotaviruses, Norwalk virus, foot and mouth disease virus, a retrovirus, Hepatitis B virus, Tobacco mosaic virus, Flock House Virus, and human Papillomavirus, and preferably a RNA phage, bacteriophage Q β , bacteriophage R17, bacteriophage M11, bacteriophage MX1, bacteriophage NL95, bacteriophage fr, bacteriophage GA, bacteriophage SP, bacteriophage MS2, bacteriophage f2, bacteriophage PP7 (for example, *see* Table 1 in Bachmann, M.F. and Zinkernagel, R.M., *Immunol. Today* 17:553-558 (1996)).

In a further embodiment, the invention utilizes genetic engineering of a virus to create a fusion between an ordered and repetitive viral envelope protein and a first

attachment site being comprised by, or alternatively or preferably being a heterologous protein, peptide, antigenic determinant or a reactive amino acid residue of choice. Other genetic manipulations known to those in the art may be included in the construction of the inventive compositions; for example, it may be desirable to
5 restrict the replication ability of the recombinant virus through genetic mutation. Furthermore, the virus used for the present invention is replication incompetent due to chemical or physical inactivation or, as indicated, due to lack of a replication competent genome. The viral protein selected for fusion to the first attachment site should have an organized and repetitive structure. Such an organized and repetitive
10 structure includes paracrystalline organizations with a spacing of 5-30 nm, preferably 5-15 nm, on the surface of the virus. The creation of this type of fusion protein will result in multiple, ordered and repetitive first attachment sites on the surface of the virus and reflect the normal organization of the native viral protein. As will be understood by those in the art, the first attachment site may be or be a part of any
15 suitable protein, polypeptide, sugar, polynucleotide, peptide (amino acid), natural or synthetic polymer, a secondary metabolite or combination thereof that may serve to specifically attach the antigen or antigenic determinant leading an ordered and repetitive antigen array.

In another embodiment of the invention, the core particle is a recombinant
20 alphavirus, and more specifically, a recombinant Sinbis virus. Alphaviruses are positive stranded RNA viruses that replicate their genomic RNA entirely in the cytoplasm of the infected cell and without a DNA intermediate (Strauss, J. and Strauss, E., *Microbiol. Rev.* 58:491-562 (1994)). Several members of the alphavirus family, Sindbis (Xiong, C. *et al.*, *Science* 243:1188-1191 (1989); Schlesinger, S.,
25 *Trends Biotechnol.* 11:18-22 (1993)), Semliki Forest Virus (SFV) (Liljeström, P. & Garoff, H., *Bio/Technology* 9:1356-1361 (1991)) and others (Davis, N.L. *et al.*, *Virology* 171:189-204 (1989)), have received considerable attention for use as virus-based expression vectors for a variety of different proteins (Lundstrom, K., *Curr. Opin. Biotechnol.* 8:578-582 (1997); Liljeström, P., *Curr. Opin. Biotechnol.* 5:495-
30 500 (1994)) and as candidates for vaccine development. Recently, a number of patents have issued directed to the use of alphaviruses for the expression of heterologous proteins and the development of vaccines (*see* U.S. Patent Nos.

5,766,602; 5,792,462; 5,739,026; 5,789,245 and 5,814,482). The construction of the alphaviral core particles of the invention may be done by means generally known in the art of recombinant DNA technology, as described by the aforementioned articles, which are incorporated herein by reference.

5 A variety of different recombinant host cells can be utilized to produce a viral-based core particle for antigen or antigenic determinant attachment. For example, alphaviruses are known to have a wide host range; Sindbis virus infects cultured mammalian, reptilian, and amphibian cells, as well as some insect cells (Clark, H., *J. Natl. Cancer Inst.* 51:645 (1973); Leake, C., *J. Gen. Virol.* 35:335 (1977); Stollar, V.
10 in THE TOGAVIRUSES, R.W. Schlesinger, Ed., Academic Press, (1980), pp.583-621). Thus, numerous recombinant host cells can be used in the practice of the invention. BHK, COS, Vero, HeLa and CHO cells are particularly suitable for the production of heterologous proteins because they have the potential to glycosylate heterologous proteins in a manner similar to human cells (Watson, E. *et al.*, *Glycobiology* 4:227,
15 (1994)) and can be selected (Zang, M. *et al.*, *Bio/Technology* 13:389 (1995)) or genetically engineered (Renner W. *et al.*, *Biotech. Bioeng.* 4:476 (1995); Lee K. *et al.* *Biotech. Bioeng.* 50:336 (1996)) to grow in serum-free medium, as well as in suspension.

Introduction of the polynucleotide vectors into host cells can be effected by
20 methods described in standard laboratory manuals (*see, e.g.*, Sambrook, J. *et al.*, eds., MOLECULAR CLONING, A LABORATORY MANUAL, 2nd. edition, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y. (1989), Chapter 9; Ausubel, F. *et al.*, eds., CURRENT PROTOCOLS IN MOLECULAR BIOLOGY, John H. Wiley & Sons, Inc. (1997), Chapter 16), including methods such as electroporation, DEAE-dextran
25 mediated transfection, transfection, microinjection, cationic lipid-mediated transfection, transduction, scrape loading, ballistic introduction, and infection. Methods for the introduction of exogenous DNA sequences into host cells are discussed in Felgner, P. *et al.*, U.S. Patent No. 5,580,859.

Packaged RNA sequences can also be used to infect host cells. These packaged
30 RNA sequences can be introduced to host cells by adding them to the culture medium. For example, the preparation of non-infective alphaviral particles is

described in a number of sources, including "Sindbis Expression System", Version C (*Invitrogen* Catalog No. K750-1).

When mammalian cells are used as recombinant host cells for the production of viral-based core particles, these cells will generally be grown in tissue culture.

5 Methods for growing cells in culture are well known in the art (*see, e.g.*, Celis, J., ed., *CELL BIOLOGY*, Academic Press, 2nd edition, (1998); Sambrook, J. *et al.*, eds., *MOLECULAR CLONING, A LABORATORY MANUAL*, 2nd. edition, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y. (1989); Ausubel, F. *et al.*, eds., *CURRENT PROTOCOLS IN MOLECULAR BIOLOGY*, John H. Wiley & Sons, Inc. (1997);

10 Freshney, R., *CULTURE OF ANIMAL CELLS*, Alan R. Liss, Inc. (1983)).

Further examples of RNA viruses suitable for use as core particle in the present invention include, but are not limited to, the following: members of the family Reoviridae, including the genus Orthoreovirus (multiple serotypes of both mammalian and avian retroviruses), the genus Orbivirus (Bluetongue virus,

15 Eugenangee virus, Kemerovo virus, African horse sickness virus, and Colorado Tick Fever virus), the genus Rotavirus (human rotavirus, Nebraska calf diarrhea virus, murine rotavirus, simian rotavirus, bovine or ovine rotavirus, avian rotavirus); the family Picomaviridae, including the genus Enterovirus (poliovirus, Coxsackie virus A and B, enteric cytopathic human orphan (ECHO) viruses, hepatitis A, C, D, E and

20 G viruses, Simian enteroviruses, Murine encephalomyelitis (ME) viruses, Poliovirus muris, Bovine enteroviruses, Porcine enteroviruses, the genus Cardiovirus (Encephalomyocarditis virus (EMC), Mengovirus), the genus Rhinovirus (Human rhinoviruses including at least 113 subtypes; other rhinoviruses), the genus Aphovirus (Foot and Mouth disease (FMDV); the family Calciviridae, including

25 Vesicular exanthema of swine virus, San Miguel sea lion virus, Feline picornavirus and Norwalk virus; the family Togaviridae, including the genus Alphavirus (Eastern equine encephalitis virus, Semliki forest virus, Sindbis virus, Chikungunya virus, O'Nyong-Nyong virus, Ross river virus, Venezuelan equine encephalitis virus, Western equine encephalitis virus), the genus Flavivirus (Mosquito borne yellow fever

30 virus, Dengue virus, Japanese encephalitis virus, St. Louis encephalitis virus, Murray Valley encephalitis virus, West Nile virus, Kunjin virus, Central European tick borne virus, Far Eastern tick borne virus, Kyasanur forest virus, Louping III virus,

Powassan virus, Omsk hemorrhagic fever virus), the genus Rubivirus (Rubella virus), the genus Pestivirus (Mucosal disease virus, Hog cholera virus, Border disease virus); the family Bunyaviridae, including the genus Bunyavirus (Bunyamwera and related viruses, California encephalitis group viruses), the genus
5 Phlebovirus (Sandfly fever Sicilian virus, Rift Valley fever virus), the genus Nairovirus (Crimean-Congo hemorrhagic fever virus, Nairobi sheep disease virus), and the genus Uukuvirus (Uukuniemi and related viruses); the family Orthomyxoviridae, including the genus Influenza virus (Influenza virus type A, many human subtypes); Swine influenza virus, and Avian and Equine Influenza
10 viruses; influenza type B (many human subtypes), and influenza type C (possible separate genus); the family paramyxoviridae, including the genus Paramyxovirus (Parainfluenza virus type 1, Sendai virus, Hemadsorption virus, Parainfluenza viruses types 2 to 5, Newcastle Disease Virus, Mumps virus), the genus Morbillivirus (Measles virus, subacute sclerosing panencephalitis virus, distemper virus,
15 Rinderpest *virus*), the genus Pneumovirus (respiratory syncytial virus (RSV), Bovine respiratory syncytial virus and Pneumonia virus of mice); forest virus, Sindbis virus, Chikungunya virus, O'Nyong-Nyong virus, Ross river virus, Venezuelan equine encephalitis virus, Western equine encephalitis virus), the genus Flavivirus (Mosquito borne yellow fever virus, Dengue virus, Japanese encephalitis virus, St. Louis
20 encephalitis virus, Murray Valley encephalitis virus, West Nile virus, Kunjin virus, Central European tick borne virus, Far Eastern tick borne virus, Kyasanur forest virus, Louping III virus, Powassan virus, Omsk hemorrhagic fever virus), the genus Rubivirus (Rubella virus), the genus Pestivirus (Mucosal disease virus, Hog cholera virus, Border disease virus); the family Bunyaviridae, including the genus Bunyavirus
25 (Bunyamwera and related viruses, California encephalitis group viruses), the genus Phlebovirus (Sandfly fever Sicilian virus, Rift Valley fever virus), the genus Nairovirus (Crimean-Congo hemorrhagic fever virus, Nairobi sheep disease virus), and the genus Uukuvirus (Uukuniemi and related viruses); the family Orthomyxoviridae, including the genus Influenza virus (Influenza virus type A,
30 many human subtypes); Swine influenza virus, and Avian and Equine Influenza viruses; influenza type B (many human subtypes), and influenza type C (possible separate genus); the family paramyxoviridae, including the genus Paramyxovirus

(Parainfluenza virus type 1, Sendai virus, Hemadsorption virus, Parainfluenza viruses types 2 to 5, Newcastle Disease Virus, Mumps virus), the genus Morbillivirus (Measles virus, subacute sclerosing panencephalitis virus, distemper virus, Rinderpest virus), the genus Pneumovirus (respiratory syncytial virus (RSV), Bovine
5 respiratory syncytial *virus* and Pneumonia virus of mice); the family Rhabdoviridae, including the genus Vesiculovirus (VSV), Chandipura virus, Flanders-Hart Park virus), the genus Lyssavirus (Rabies virus), fish Rhabdoviruses and, filoviruses (Marburg virus and Ebola virus); the family Arenaviridae, including Lymphocytic choriomeningitis virus (LCM), Tacaribe virus complex, and Lassa virus; the family
10 Coronaviridae, including Infectious Bronchitis Virus (IBV), Mouse Hepatitis virus, Human enteric corona virus, and Feline infectious peritonitis (Feline coronavirus).

Illustrative DNA viruses that may be used as core particle include, but are not limited to: the family Poxviridae, including the genus Orthopoxvirus (Variola major, Variola minor, Monkey pox Vaccinia, Cowpox, Buffalopox, Rabbitpox, Ectromelia),
15 the genus Leporipoxvirus (Myxoma, Fibroma), the genus Avipoxvirus (Fowlpox, other avian poxvirus), the genus Capripoxvirus (sheeppox, goatpox), the genus Suipoxvirus (Swinepox), the genus Parapoxvirus (contagious postular dermatitis virus, pseudocowpox, bovine papular stomatitis virus); the family Iridoviridae (African swine fever virus, Frog viruses 2 and 3, Lymphocystis virus of fish); the
20 family Herpesviridae, including the alpha-Herpesviruses (Herpes Simplex Types 1 and 2, Varicella-Zoster, Equine abortion virus, Equine herpes virus 2 and 3, pseudorabies virus, infectious bovine keratoconjunctivitis virus, infectious bovine rhinotracheitis virus, feline rhinotracheitis virus, infectious laryngotracheitis virus) the Beta-herpesviruses (Human cytomegalovirus and cytomegaloviruses of swine,
25 monkeys and rodents); the gamma-herpesviruses (Epstein-Barr virus (EBV), Marek's disease virus, Herpes saimiri, Herpesvirus ateles, Herpesvirus sylvilagus, guinea pig herpes virus, Lucke tumor virus); the family Adenoviridae, including the genus Mastadenovirus (Human subgroups A, B, C, D and E and ungrouped; simian adenoviruses (at least 23 serotypes), infectious canine hepatitis, and adenoviruses of
30 cattle, pigs, sheep, frogs and many other species, the genus Aviadenovirus (Avian adenoviruses); and non-cultivable adenoviruses; the family Papoviridae, including the genus Papillomavirus (Human papilloma viruses, bovine papilloma viruses,

Shope rabbit papilloma virus, and various pathogenic papilloma viruses of other species), the genus Polyomavirus (polyomavirus, Simian vacuolating agent (SV-40), Rabbit vacuolating agent (RKV), K virus, BK virus, JC virus, and other primate polyoma viruses such as Lymphotropic papilloma virus); the family Parvoviridae including the genus Adeno-associated viruses, the genus Parvovirus (Feline panleukopenia virus, bovine parvovirus, canine parvovirus, Aleutian mink disease virus, etc.). Finally, DNA viruses may include viruses such as chronic infectious neuropathic agents (CHINA virus).

In other embodiments, a bacterial pilin, a subportion of a bacterial pilin, or a fusion protein which contains either a bacterial pilin or subportion thereof is used to prepare compositions and vaccine compositions, respectively, of the invention. Examples of pilin proteins include pilins produced by *Escherichia coli*, *Haemophilus influenzae*, *Neisseria meningitidis*, *Neisseria gonorrhoeae*, *Caulobacter crescentus*, *Pseudomonas stutzeri*, and *Pseudomonas aeruginosa*. The amino acid sequences of pilin proteins suitable for use with the present invention include those set out in GenBank reports AJ000636, AJ132364, AF229646, AF051814, AF051815, and X00981, the entire disclosures of which are incorporated herein by reference.

Bacterial pilin proteins are generally processed to remove N-terminal leader sequences prior to export of the proteins into the bacterial periplasm. Further, as one skilled in the art would recognize, bacterial pilin proteins used to prepare compositions and vaccine compositions, respectively, of the invention will generally not have the naturally present leader sequence.

One specific example of a pilin protein suitable for use in the present invention is the P-pilin of *E. coli* (GenBank report AF237482 (SEQ ID NO:1)). An example of a Type-1 *E. coli* pilin suitable for use with the invention is a pilin having the amino acid sequence set out in GenBank report P04128 (SEQ ID NO:2), which is encoded by nucleic acid having the nucleotide sequence set out in GenBank report M27603 (SEQ ID NO:3). The entire disclosures of these GenBank reports are incorporated herein by reference. Again, the mature form of the above referenced protein would generally be used to prepare compositions and vaccine compositions, respectively, of the invention.

Bacterial pilins or pilin subportions suitable for use in the practice of the present invention will generally be able to associate to form ordered and repetitive antigen arrays.

Methods for preparing pili and pilus-like structures *in vitro* are known in the art. Bullitt *et al.*, *Proc. Natl. Acad. Sci. USA* 93:12890-12895 (1996), for example, describe the *in vitro* reconstitution of *E. coli* P-pili subunits. Furthermore, Eshdat *et al.*, *J. Bacteriol.* 148:308-314 (1981) describe methods suitable for dissociating Type-1 pili of *E. coli* and the reconstitution of pili. In brief, these methods are as follows: pili are dissociated by incubation at 37°C in saturated guanidine hydrochloride. Pilin proteins are then purified by chromatography, after which pilin dimers are formed by dialysis against 5 mM tris(hydroxymethyl) aminomethane hydrochloride (pH 8.0). Eshdat *et al.* also found that pilin dimers reassemble to form pili upon dialysis against the 5 mM tris(hydroxymethyl) aminomethane (pH 8.0) containing 5 mM MgCl₂.

Further, using, for example, conventional genetic engineering and protein modification methods, pilin proteins may be modified to contain a first attachment site to which an antigen or antigenic determinant is linked through a second attachment site. Alternatively, antigens or antigenic determinants can be directly linked through a second attachment site to amino acid residues which are naturally resident in these proteins. These modified pilin proteins may then be used in vaccine compositions of the invention.

Bacterial pilin proteins used to prepare compositions and vaccine compositions, respectively, of the invention may be modified in a manner similar to that described herein for HBcAg. For example, cysteine and lysine residues may be either deleted or substituted with other amino acid residues and first attachment sites may be added to these proteins. Further, pilin proteins may either be expressed in modified form or may be chemically modified after expression. Similarly, intact pili may be harvested from bacteria and then modified chemically.

In another embodiment, pili or pilus-like structures are harvested from bacteria (*e.g.*, *E. coli*) and used to form compositions and vaccine compositions of the invention. One example of pili suitable for preparing compositions and vaccine

compositions is the Type-1 pilus of *E. coli*, which is formed from pilin monomers having the amino acid sequence set out in SEQ ID NO:2.

A number of methods for harvesting bacterial pili are known in the art. Bullitt and Makowski (*Biophys. J.* 74:623-632 (1998)), for example, describe a pilus
5 purification method for harvesting P-pili from *E. coli*. According to this method, pili are sheared from hyperpilated *E. coli* containing a P-pilus plasmid and purified by cycles of solubilization and MgCl₂ (1.0 M) precipitation.

Once harvested, pili or pilus-like structures may be modified in a variety of ways. For example, a first attachment site can be added to the pili to which antigens
10 or antigen determinants may be attached through a second attachment site. In other words, bacterial pili or pilus-like structures can be harvested and modified to lead to ordered and repetitive antigen arrays.

Antigens or antigenic determinants could be linked to naturally occurring cysteine residues or lysine residues present in Pili or pilus-like structures. In such
15 instances, the high order and repetitiveness of a naturally occurring amino acid residue would guide the coupling of the antigens or antigenic determinants to the pili or pilus-like structures. For example, the pili or pilus-like structures could be linked to the second attachment sites of the antigens or antigenic determinants using a heterobifunctional cross-linking agent.

20 When structures which are naturally synthesized by organisms (*e.g.*, pili) are used to prepare compositions and vaccine compositions of the invention, it will often be advantageous to genetically engineer these organisms so that they produce structures having desirable characteristics. For example, when Type-1 pili of *E. coli* are used, the *E. coli* from which these pili are harvested may be modified so as to
25 produce structures with specific characteristics. Examples of possible modifications of pilin proteins include the insertion of one or more lysine residues, the deletion or substitution of one or more of the naturally resident lysine residues, and the deletion or substitution of one or more naturally resident cysteine residues (*e.g.*, the cysteine residues at positions 44 and 84 in SEQ ID NO:2).

30 Further, additional modifications can be made to pilin genes which result in the expression products containing a first attachment site other than a lysine residue (*e.g.*, a *FOS* or *JUN* domain). Of course, suitable first attachment sites will generally

be limited to those which do not prevent pilin proteins from forming pili or pilus-like structures suitable for use in vaccine compositions of the invention.

Pilin genes which naturally reside in bacterial cells can be modified *in vivo* (e.g., by homologous recombination) or pilin genes with particular characteristics can
5 be inserted into these cells. For examples, pilin genes could be introduced into bacterial cells as a component of either a replicable cloning vector or a vector which inserts into the bacterial chromosome. The inserted pilin genes may also be linked to expression regulatory control sequences (e.g., a *lac* operator).

In most instances, the pili or pilus-like structures used in compositions and
10 vaccine compositions, respectively, of the invention will be composed of single type of a pilin subunit. Pili or pilus-like structures composed of identical subunits will generally be used because they are expected to form structures which present highly ordered and repetitive antigen arrays.

However, the compositions of the invention also include compositions and
15 vaccines comprising pili or pilus-like structures formed from heterogenous pilin subunits. The pilin subunits which form these pili or pilus-like structures can be expressed from genes naturally resident in the bacterial cell or may be introduced into the cells. When a naturally resident pilin gene and an introduced gene are both expressed in a cell which forms pili or pilus-like structures, the result will generally
20 be structures formed from a mixture of these pilin proteins. Further, when two or more pilin genes are expressed in a bacterial cell, the relative expression of each pilin gene will typically be the factor which determines the ratio of the different pilin subunits in the pili or pilus-like structures.

When pili or pilus-like structures having a particular composition of mixed
25 pilin subunits is desired, the expression of at least one of the pilin genes can be regulated by a heterologous, inducible promoter. Such promoters, as well as other genetic elements, can be used to regulate the relative amounts of different pilin subunits produced in the bacterial cell and, hence, the composition of the pili or pilus-like structures.

30 In additional, the antigen or antigenic determinant can be linked to bacterial pili or pilus-like structures by a bond which is not a peptide bond, bacterial cells which produce pili or pilus-like structures used in the compositions of the invention

can be genetically engineered to generate pilin proteins which are fused to an antigen or antigenic determinant. Such fusion proteins which form pili or pilus-like structures are suitable for use in vaccine compositions of the invention.

In a preferred embodiment, the core particle is a virus-like particle, wherein the virus-like particle is a recombinant virus-like particle. The skilled artisan can produce VLPs using recombinant DNA technology and virus coding sequences which are readily available to the public. For example, the coding sequence of a virus envelope or core protein can be engineered for expression in a baculovirus expression vector using a commercially available baculovirus vector, under the regulatory control of a virus promoter, with appropriate modifications of the sequence to allow functional linkage of the coding sequence to the regulatory sequence. The coding sequence of a virus envelope or core protein can also be engineered for expression in a bacterial expression vector, for example.

Examples of VLPs include, but are not limited to, the capsid proteins of Hepatitis B virus (Ulrich, *et al.*, *Virus Res.* 50:141-182 (1998)), measles virus (Warnes, *et al.*, *Gene* 160:173-178 (1995)), Sindbis virus, rotavirus (US 5,071,651 and US 5,374,426), foot-and-mouth-disease virus (Twomey, *et al.*, *Vaccine* 13:1603-1610, (1995)), Norwalk virus (Jiang, X., *et al.*, *Science* 250:1580-1583 (1990); Matsui, S.M., *et al.*, *J. Clin. Invest.* 87:1456-1461 (1991)), the retroviral GAG protein (WO 96/30523), the retrotransposon Ty protein p1, the surface protein of Hepatitis B virus (WO 92/11291), human papilloma virus (WO 98/15631), RNA phages, Ty, fr-phage, GA-phage, AP205-phage and Q β -phage.

As will be readily apparent to those skilled in the art, the VLP of the invention is not limited to any specific form. The particle can be synthesized chemically or through a biological process, which can be natural or non-natural. By way of example, this type of embodiment includes a virus-like particle or a recombinant form thereof.

In a more specific embodiment, the VLP can comprise, or alternatively essentially consist of, or alternatively consist of recombinant polypeptides, or fragments thereof, being selected from recombinant polypeptides of Rotavirus, recombinant polypeptides of Norwalk virus, recombinant polypeptides of Alphavirus, recombinant polypeptides of Foot and Mouth Disease virus, recombinant

polypeptides of measles virus, recombinant polypeptides of Sindbis virus, recombinant polypeptides of Polyoma virus, recombinant polypeptides of Retrovirus, recombinant polypeptides of Hepatitis B virus (*e.g.*, a HBcAg), recombinant polypeptides of Tobacco mosaic virus, recombinant polypeptides of Flock House
5 Virus, recombinant polypeptides of human Papillomavirus, recombinant polypeptides of bacteriophages, recombinant polypeptides of RNA phages, recombinant polypeptides of Ty, recombinant polypeptides of fr-phage, recombinant polypeptides of GA-phage and recombinant polypeptides of Q β -phage. The virus-like particle can further comprise, or alternatively essentially consist of, or
10 alternatively consist of, one or more fragments of such polypeptides, as well as variants of such polypeptides. Variants of polypeptides can share, for example, at least 80%, 85%, 90%, 95%, 97%, or 99% identity at the amino acid level with their wild-type counterparts.

In a preferred embodiment, the virus-like particle comprises, consists
15 essentially of, or alternatively consists of recombinant proteins, or fragments thereof, of a RNA-phage. Preferably, the RNA-phage is selected from the group consisting of a) bacteriophage Q β ; b) bacteriophage R17; c) bacteriophage fr; d) bacteriophage GA; e) bacteriophage SP; f) bacteriophage MS2; g) bacteriophage M11; h) bacteriophage MX1; i) bacteriophage NL95; k) bacteriophage f2; l) bacteriophage
20 PP7, and m) bacteriophage AP205.

In another preferred embodiment of the present invention, the virus-like particle comprises, or alternatively consists essentially of, or alternatively consists of recombinant proteins, or fragments thereof, of the RNA-bacteriophage Q β or of the RNA-bacteriophage fr, or of the RNA-bacteriophage AP205.

25 In a further preferred embodiment of the present invention, the recombinant proteins comprise, or alternatively consist essentially of, or alternatively consist of coat proteins of RNA phages.

RNA-phage coat proteins forming capsids or VLPs, or fragments of the bacteriophage coat proteins compatible with self-assembly into a capsid or a VLP,
30 are, therefore, further preferred embodiments of the present invention. Bacteriophage Q β coat proteins, for example, can be expressed recombinantly in *E.*

coli. Further, upon such expression these proteins spontaneously form capsids. Additionally, these capsids form a structure with an inherent repetitive organization.

Specific preferred examples of bacteriophage coat proteins which can be used to prepare compositions of the invention include the coat proteins of RNA
5 bacteriophages such as bacteriophage Q β (SEQ ID NO:4; PIR Database, Accession No. VCBPQ β referring to Q β CP and SEQ ID NO: 5; Accession No. AAA16663 referring to Q β A1 protein), bacteriophage R17 (SEQ ID NO:6; PIR Accession No. VCBPR7), bacteriophage fr (SEQ ID NO:7; PIR Accession No. VCBPFR), bacteriophage GA (SEQ ID NO:8; GenBank Accession No. NP-040754),
10 bacteriophage SP (SEQ ID NO:9; GenBank Accession No. CAA30374 referring to SP CP and SEQ ID NO: 10; Accession No. NP 695026 referring to SP A1 protein), bacteriophage MS2 (SEQ ID NO:11; PIR Accession No. VCBPM2), bacteriophage M11 (SEQ ID NO:12; GenBank Accession No. AAC06250), bacteriophage MX1 (SEQ ID NO:13; GenBank Accession No. AAC14699), bacteriophage NL95 (SEQ
15 ID NO:14; GenBank Accession No. AAC14704), bacteriophage f2 (SEQ ID NO: 15; GenBank Accession No. P03611), bacteriophage PP7 (SEQ ID NO: 16), and bacteriophage AP205 (SEQ ID NO: 28). Furthermore, the A1 protein of bacteriophage Q β (SEQ ID NO: 5) or C-terminal truncated forms missing as much as 100, 150 or 180 amino acids from its C-terminus may be incorporated in a capsid
20 assembly of Q β coat proteins. Generally, the percentage of Q β A1 protein relative to Q β CP in the capsid assembly will be limited, in order to ensure capsid formation.

Q β coat protein has also been found to self-assemble into capsids when expressed in *E. coli* (Kozlovska TM. *et al.*, *GENE* 137: 133-137 (1993)). The obtained capsids or virus-like particles showed an icosahedral phage-like capsid
25 structure with a diameter of 25 nm and T=3 quasi symmetry. Further, the crystal structure of phage Q β has been solved. The capsid contains 180 copies of the coat protein, which are linked in covalent pentamers and hexamers by disulfide bridges (Golmohammadi, R. *et al.*, *Structure* 4: 543-5554 (1996)) leading to a remarkable stability of the capsid of Q β coat protein. Capsids or VLPs made from recombinant
30 Q β coat protein may contain, however, subunits not linked via disulfide links to other subunits within the capsid, or incompletely linked. However, typically more than about 80% of the subunits are linked via disulfide bridges to each other within the

VLP. Thus, upon loading recombinant Q β capsid on non-reducing SDS-PAGE, bands corresponding to monomeric Q β coat protein as well as bands corresponding to the hexamer or pentamer of Q β coat protein are visible. Incompletely disulfide-linked subunits could appear as dimer, trimer or even tetramer bands in non-reducing SDS-PAGE. Q β capsid protein also shows unusual resistance to organic solvents and denaturing agents. Surprisingly, we have observed that DMSO and acetonitrile concentrations as high as 30%, and Guanidinium concentrations as high as 1 M do not affect the stability of the capsid. The high stability of the capsid of Q β coat protein is an advantageous feature, in particular, for its use in immunization and vaccination of mammals and humans in accordance of the present invention.

Upon expression in *E. coli*, the N-terminal methionine of Q β coat protein is usually removed, as we observed by N-terminal Edman sequencing as described in Stoll, E. et al. J. Biol. Chem. 252:990-993 (1977). VLP composed from Q β coat proteins where the N-terminal methionine has not been removed, or VLPs comprising a mixture of Q β coat proteins where the N-terminal methionine is either cleaved or present are also within the scope of the present invention.

Further preferred virus-like particles of RNA-phages, in particular of Q β , in accordance of this invention are disclosed in WO 02/056905, the disclosure of which is herewith incorporated by reference in its entirety.

Further RNA phage coat proteins have also been shown to self-assemble upon expression in a bacterial host (Kastelein, RA. *et al.*, *Gene* 23: 245-254 (1983), Kozlovskaya, TM. *et al.*, *Dokl. Akad. Nauk SSSR* 287: 452-455 (1986), Adhin, MR. *et al.*, *Virology* 170: 238-242 (1989), Ni, CZ., *et al.*, *Protein Sci.* 5: 2485-2493 (1996), Priano, C. *et al.*, *J. Mol. Biol.* 249: 283-297 (1995)). The Q β phage capsid contains, in addition to the coat protein, the so called read-through protein A1 and the maturation protein A2. A1 is generated by suppression at the UGA stop codon and has a length of 329 aa. The capsid of phage Q β recombinant coat protein used in the invention is devoid of the A2 lysis protein, and contains RNA from the host. The coat protein of RNA phages is an RNA binding protein, and interacts with the stem loop of the ribosomal binding site of the replicase gene acting as a translational repressor during the life cycle of the virus. The sequence and structural elements of the interaction are known (Witherell, GW. & Uhlenbeck, OC. *Biochemistry* 28: 71-

76 (1989); Lim F. et al., *J. Biol. Chem.* 271: 31839-31845 (1996)). The stem loop and RNA in general are known to be involved in the virus assembly (Golmohammadi, R. et al., *Structure* 4: 543-5554 (1996)).

In a further preferred embodiment of the present invention, the virus-like particle comprises, or alternatively consists essentially of, or alternatively consists of recombinant proteins, or fragments thereof, of a RNA-phage, wherein the recombinant proteins comprise, alternatively consist essentially of or alternatively consist of mutant coat proteins of a RNA phage, preferably of mutant coat proteins of the RNA phages mentioned above. In another preferred embodiment, the mutant coat proteins of the RNA phage have been modified by removal of at least one, or alternatively at least two, lysine residue by way of substitution, or by addition of at least one lysine residue by way of substitution; alternatively, the mutant coat proteins of the RNA phage have been modified by deletion of at least one, or alternatively at least two, lysine residue, or by addition of at least one lysine residue by way of insertion. The deletion, substitution or addition of at least one lysine residue allows varying the degree of coupling, i.e. the amount of A β 1-6 peptides per subunits of the VLP of the RNA-phages, in particular, to match and tailor the requirements of the vaccine.

In another preferred embodiment, the virus-like particle comprises, or alternatively consists essentially of, or alternatively consists of recombinant proteins, or fragments thereof, of the RNA-bacteriophage Q β , wherein the recombinant proteins comprise, or alternatively consist essentially of, or alternatively consist of coat proteins having an amino acid sequence of SEQ ID NO:4, or a mixture of coat proteins having amino acid sequences of SEQ ID NO:4 and of SEQ ID NO: 5 or mutants of SEQ ID NO: 5 and wherein the N-terminal methionine is preferably cleaved.

In a further preferred embodiment of the present invention, the virus-like particle comprises, consists essentially of or alternatively consists of recombinant proteins of Q β , or fragments thereof, wherein the recombinant proteins comprise, or alternatively consist essentially of, or alternatively consist of mutant Q β coat proteins. In another preferred embodiment, these mutant coat proteins have been modified by removal of at least one lysine residue by way of substitution, or by

addition of at least one lysine residue by way of substitution. Alternatively, these mutant coat proteins have been modified by deletion of at least one lysine residue, or by addition of at least one lysine residue by way of insertion.

Four lysine residues are exposed on the surface of the capsid of Q β coat protein. Q β mutants, for which exposed lysine residues are replaced by arginines can also be used for the present invention. The following Q β coat protein mutants and mutant Q β VLPs can, thus, be used in the practice of the invention: "Q β -240" (Lys13-Arg; SEQ ID NO:17), "Q β -243" (Asn 10-Lys; SEQ ID NO:18), "Q β -250" (Lys 2-Arg, Lys13-Arg; SEQ ID NO:19), "Q β -251" (SEQ ID NO:20) and "Q β -259" (Lys 2-Arg, Lys16-Arg; SEQ ID NO:21). Thus, in further preferred embodiment of the present invention, the virus-like particle comprises, consists essentially of or alternatively consists of recombinant proteins of mutant Q β coat proteins, which comprise proteins having an amino acid sequence selected from the group of a) the amino acid sequence of SEQ ID NO: 17; b) the amino acid sequence of SEQ ID NO:18; c) the amino acid sequence of SEQ ID NO: 19; d) the amino acid sequence of SEQ ID NO:20; and e) the amino acid sequence of SEQ ID NO: 21. The construction, expression and purification of the above indicated Q β coat proteins, mutant Q β coat protein VLPs and capsids, respectively, are described in WO 02/056905. In particular is hereby referred to Example 18 of above mentioned application.

In a further preferred embodiment of the present invention, the virus-like particle comprises, or alternatively consists essentially of, or alternatively consists of recombinant proteins of Q β , or fragments thereof, wherein the recombinant proteins comprise, consist essentially of or alternatively consist of a mixture of either one of the foregoing Q β mutants and the corresponding A1 protein.

In a further preferred embodiment of the present invention, the virus-like particle comprises, or alternatively essentially consists of, or alternatively consists of recombinant proteins, or fragments thereof, of RNA-phage AP205.

The AP205 genome consists of a maturation protein, a coat protein, a replicase and two open reading frames not present in related phages; a lysis gene and an open reading frame playing a role in the translation of the maturation gene (Klovins, J., *et al.*, *J. Gen. Virol.* 83: 1523-33 (2002)). AP205 coat protein can be expressed from

plasmid pAP283-58 (SEQ ID NO: 27), which is a derivative of pQb10 (Kozlovskaya, T. M., *et al.*, *Gene* 137:133-37 (1993)), and which contains an AP205 ribosomal binding site. Alternatively, AP205 coat protein may be cloned into pQb185, downstream of the ribosomal binding site present in the vector. Both approaches lead to expression of the protein and formation of capsids as described in the co-pending US provisional patent application with the title "Molecular Antigen Arrays" (Atty. Docket No. 1700.0310000) and having been filed on July 17, 2002, which is incorporated by reference in its entirety. Vectors pQb10 and pQb185 are vectors derived from pGEM vector, and expression of the cloned genes in these vectors is controlled by the *trp* promoter (Kozlovskaya, T. M., *et al.*, *Gene* 137:133-37 (1993)). Plasmid pAP283-58 (SEQ ID NO:27) comprises a putative AP205 ribosomal binding site in the following sequence, which is downstream of the XbaI site, and immediately upstream of the ATG start codon of the AP205 coat protein:

*tctaga*ATTTTCTGCGCACCCAT

CCCCGGGTGGCGCCCAAAGTGAGGAAAATC*Catg* (SEQ ID NO:57). The vector pQb185 comprises a Shine Delagarno sequence downstream from the XbaI site and upstream of the start codon (*tctaga*TTAACCCAACGCGTAGGAGTCAGGCC*Catg* (SEQ ID NO:58), Shine Delagarno sequence underlined).

In a further preferred embodiment of the present invention, the virus-like particle comprises, or alternatively essentially consists of, or alternatively consists of recombinant coat proteins, or fragments thereof, of the RNA-phage AP205.

This preferred embodiment of the present invention, thus, comprises AP205 coat proteins that form capsids. Such proteins are recombinantly expressed, or prepared from natural sources. AP205 coat proteins produced in bacteria spontaneously form capsids, as evidenced by Electron Microscopy (EM) and immunodiffusion. The structural properties of the capsid formed by the AP205 coat protein (SEQ ID NO: 28) and those formed by the coat protein of the AP205 RNA phage are nearly indistinguishable when seen in EM. AP205 VLPs are highly immunogenic, and can be linked with antigens and/or antigenic determinants to generate vaccine constructs displaying the antigens and/or antigenic determinants oriented in a repetitive manner. High titers are elicited against the so displayed

antigens showing that bound antigens and/or antigenic determinants are accessible for interacting with antibody molecules and are immunogenic.

In a further preferred embodiment of the present invention, the virus-like particle comprises, or alternatively essentially consists of, or alternatively consists of
5 recombinant mutant coat proteins, or fragments thereof, of the RNA-phage AP205.

Assembly-competent mutant forms of AP205 VLPs, including AP205 coat protein with the substitution of proline at amino acid 5 to threonine (SEQ ID NO: 29), may also be used in the practice of the invention and leads to a further preferred embodiment of the invention. These VLPs, AP205 VLPs derived from natural
10 sources, or AP205 viral particles, may be bound to antigens to produce ordered repetitive arrays of the antigens in accordance with the present invention.

AP205 P5-T mutant coat protein can be expressed from plasmid pAP281-32 (SEQ ID No. 30), which is derived directly from pQb185, and which contains the mutant AP205 coat protein gene instead of the Q β coat protein gene. Vectors for
15 expression of the AP205 coat protein are transfected into *E. coli* for expression of the AP205 coat protein.

Methods for expression of the coat protein and the mutant coat protein, respectively, leading to the self-assembly into VLPs are described in Example 1. Suitable *E. coli* strains include, but are not limited to, *E. coli* K802, JM 109, RR1.
20 Suitable vectors and strains and combinations thereof can be identified by testing expression of the coat protein and mutant coat protein, respectively, by SDS-PAGE and capsid formation and assembly by optionally first purifying the capsids by gel filtration and subsequently testing them in an immunodiffusion assay (Ouchterlony test) or Electron Microscopy (Kozlovska, T. M., *et al.*, *Gene* 137:133-37 (1993)).

25 AP205 coat proteins expressed from the vectors pAP283-58 and pAP281-32 may be devoid of the initial Methionine amino-acid, due to processing in the cytoplasm of *E. coli*. Cleaved, uncleaved forms of AP205 VLP or mixtures thereof are further preferred embodiments of the invention.

In a further preferred embodiment of the present invention, the virus-like
30 particle comprises, or alternatively essentially consists of, or alternatively consists of a mixture of recombinant coat proteins, or fragments thereof, of the RNA-phage

AP205 and of recombinant mutant coat proteins, or fragments thereof, of the RNA-phage AP205.

In a further preferred embodiment of the present invention, the virus-like particle comprises, or alternatively essentially consists of, or alternatively consists of
5 fragments of recombinant coat proteins or recombinant mutant coat proteins of the RNA-phage AP205.

Recombinant AP205 coat protein fragments capable of assembling into a VLP and a capsid, respectively are also useful in the practice of the invention. These fragments may be generated by deletion, either internally or at the termini of the coat
10 protein and mutant coat protein, respectively. Insertions in the coat protein and mutant coat protein sequence or fusions of antigen sequences to the coat protein and mutant coat protein sequence, and compatible with assembly into a VLP, are further embodiments of the invention and lead to chimeric AP205 coat proteins, and particles, respectively. The outcome of insertions, deletions and fusions to the coat
15 protein sequence and whether it is compatible with assembly into a VLP can be determined by electron microscopy.

The particles formed by the AP205 coat protein, coat protein fragments and chimeric coat proteins described above, can be isolated in pure form by a combination of fractionation steps by precipitation and of purification steps by gel
20 filtration using *e.g.* Sepharose CL-4B, Sepharose CL-2B, Sepharose CL-6B columns and combinations thereof. Other methods of isolating virus-like particles are known in the art, and may be used to isolate the virus-like particles (VLPs) of bacteriophage AP205. For example, the use of ultracentrifugation to isolate VLPs of the yeast retrotransposon Ty is described in U.S. Patent No. 4,918,166, which is incorporated
25 by reference herein in its entirety.

The crystal structure of several RNA bacteriophages has been determined (Golmohammadi, R. *et al.*, *Structure* 4:543-554 (1996)). Using such information, surface exposed residues can be identified and, thus, RNA-phage coat proteins can be modified such that one or more reactive amino acid residues can be inserted by
30 way of insertion or substitution. As a consequence, those modified forms of bacteriophage coat proteins can also be used for the present invention. Thus, variants of proteins which form capsids or capsid-like structures (*e.g.*, coat proteins of

bacteriophage Q β , bacteriophage R17, bacteriophage fr, bacteriophage GA, bacteriophage SP, bacteriophage AP205, and bacteriophage MS2) can also be used to prepare compositions of the present invention.

Although the sequence of the variants proteins discussed above will differ from
5 their wild-type counterparts, these variant proteins will generally retain the ability to form capsids or capsid-like structures. Thus, the invention further includes compositions and vaccine compositions, respectively, which further includes variants of proteins which form capsids or capsid-like structures, as well as methods for preparing such compositions and vaccine compositions, respectively, individual
10 protein subunits used to prepare such compositions, and nucleic acid molecules which encode these protein subunits. Thus, included within the scope of the invention are variant forms of wild-type proteins which form capsids or capsid-like structures and retain the ability to associate and form capsids or capsid-like structures.

15 As a result, the invention further includes compositions and vaccine compositions, respectively, comprising proteins, which comprise, or alternatively consist essentially of, or alternatively consist of amino acid sequences which are at least 80%, 85%, 90%, 95%, 97%, or 99% identical to wild-type proteins which form ordered arrays and have an inherent repetitive structure, respectively.

20 Further included within the scope of the invention are nucleic acid molecules which encode proteins used to prepare compositions of the present invention.

In other embodiments, the invention further includes compositions comprising proteins, which comprise, or alternatively consist essentially of, or alternatively consist of amino acid sequences which are at least 80%, 85%, 90%, 95%, 97%, or
25 99% identical to any of the amino acid sequences shown in SEQ ID NOs:4-21.

Proteins suitable for use in the present invention also include C-terminal truncation mutants of proteins which form capsids or capsid-like structures, or VLPs. Specific examples of such truncation mutants include proteins having an amino acid sequence shown in any of SEQ ID NOs:4-21 where 1, 2, 5, 7, 9, 10, 12, 14, 15, or 17
30 amino acids have been removed from the C-terminus. Typically, these C-terminal truncation mutants will retain the ability to form capsids or capsid-like structures.

Further proteins suitable for use in the present invention also include N-terminal truncation mutants of proteins which form capsids or capsid-like structures. Specific examples of such truncation mutants include proteins having an amino acid sequence shown in any of SEQ ID NOs:4-21 where 1, 2, 5, 7, 9, 10, 12,
5 14, 15, or 17 amino acids have been removed from the N-terminus. Typically, these N-terminal truncation mutants will retain the ability to form capsids or capsid-like structures.

Additional proteins suitable for use in the present invention include N- and C-terminal truncation mutants which form capsids or capsid-like structures. Suitable
10 truncation mutants include proteins having an amino acid sequence shown in any of SEQ ID NOs:4-21 where 1, 2, 5, 7, 9, 10, 12, 14, 15, or 17 amino acids have been removed from the N-terminus and 1, 2, 5, 7, 9, 10, 12, 14, 15, or 17 amino acids have been removed from the C-terminus. Typically, these N-terminal and C-terminal truncation mutants will retain the ability to form capsids or capsid-like structures.

15 The invention further includes compositions comprising proteins which comprise, or alternatively consist essentially of, or alternatively consist of, amino acid sequences which are at least 80%, 85%, 90%, 95%, 97%, or 99% identical to the above described truncation mutants.

The invention thus includes compositions and vaccine compositions prepared
20 from proteins which form capsids or VLPs, methods for preparing these compositions from individual protein subunits and VLPs or capsids, methods for preparing these individual protein subunits, nucleic acid molecules which encode these subunits, and methods for vaccinating and/or eliciting immunological responses in individuals using these compositions of the present invention.

25 As previously stated, the invention includes virus-like particles or recombinant forms thereof. In one further preferred embodiment, the particles used in compositions of the invention are composed of a Hepatitis B core protein (HBcAg) or a fragment of a HBcAg. In a further embodiment, the particles used in compositions of the invention are composed of a Hepatitis B core protein (HBcAg)
30 or a fragment of a HBcAg protein, which has been modified to either eliminate or reduce the number of free cysteine residues. Zhou *et al.* (*J. Virol.* 66:5393-5398 (1992)) demonstrated that HBcAgS which have been modified to remove the

naturally resident cysteine residues retain the ability to associate and form capsids. Thus, VLPs suitable for use in compositions of the invention include those comprising modified HBcAgs, or fragments thereof, in which one or more of the naturally resident cysteine residues have been either deleted or substituted with
5 another amino acid residue (*e.g.*, a serine residue).

The HBcAg is a protein generated by the processing of a Hepatitis B core antigen precursor protein. A number of isotypes of the HBcAg have been identified and their amino acids sequences are readily available to those skilled in the art. In most instances, compositions and vaccine compositions, respectively, of the
10 invention will be prepared using the processed form of a HBcAg (*i.e.*, a HBcAg from which the N-terminal leader sequence of the Hepatitis B core antigen precursor protein have been removed).

Further, when HBcAgs are produced under conditions where processing will not occur, the HBcAgs will generally be expressed in "processed" form. For
15 example, when an *E. coli* expression system directing expression of the protein to the cytoplasm is used to produce HBcAgs of the invention, these proteins will generally be expressed such that the N-terminal leader sequence of the Hepatitis B core antigen precursor protein is not present.

The preparation of Hepatitis B virus-like particles, which can be used for the
20 present invention, is disclosed, for example, in WO 00/32227, and hereby in particular in Examples 17 to 19 and 21 to 24, as well as in WO 01/85208, and hereby in particular in Examples 17 to 19, 21 to 24, 31 and 41, and in WO 02/056905. For the latter application, it is in particular referred to Example 23, 24, 31 and 51. All three documents are explicitly incorporated herein by reference.

25 The present invention also includes HBcAg variants which have been modified to delete or substitute one or more additional cysteine residues. It is known in the art that free cysteine residues can be involved in a number of chemical side reactions. These side reactions include disulfide exchanges, reaction with chemical substances or metabolites that are, for example, injected or formed in a combination therapy
30 with other substances, or direct oxidation and reaction with nucleotides upon exposure to UV light. Toxic adducts could thus be generated, especially considering the fact that HBcAgs have a strong tendency to bind nucleic acids. The toxic adducts

would thus be distributed between a multiplicity of species, which individually may each be present at low concentration, but reach toxic levels when together.

In view of the above, one advantage to the use of HBcAgs in vaccine compositions which have been modified to remove naturally resident cysteine residues is that sites to which toxic species can bind when antigens or antigenic determinants are attached would be reduced in number or eliminated altogether.

A number of naturally occurring HBcAg variants suitable for use in the practice of the present invention have been identified. Yuan *et al.*, (*J. Virol.* 73:10122-10128 (1999)), for example, describe variants in which the isoleucine residue at position corresponding to position 97 in SEQ ID NO:22 is replaced with either a leucine residue or a phenylalanine residue. The amino acid sequences of a number of HBcAg variants, as well as several Hepatitis B core antigen precursor variants, are disclosed in GenBank reports AAF121240, AF121239, X85297, X02496, X85305, X85303, AF151735, X85259, X85286, X85260, X85317, X85298, AF043593, M20706, X85295, X80925, X85284, X85275, X72702, X85291, X65258, X85302, M32138, X85293, X85315, U95551, X85256, X85316, X85296, AB033559, X59795, X85299, X85307, X65257, X85311, X85301 (SEQ ID NO:23), X85314, X85287, X85272, X85319, AB010289, X85285, AB010289, AF121242, M90520 (SEQ ID NO:24), P03153, AF110999, and M95589, the disclosures of each of which are incorporated herein by reference. The sequences of the hereinabove mentioned Hepatitis B core antigen precursor variants are further disclosed in WO 01/85208 in SEQ ID NOs: 89 – 138. These HBcAg variants differ in amino acid sequence at a number of positions, including amino acid residues which corresponds to the amino acid residues located at positions 12, 13, 21, 22, 24, 29, 32, 33, 35, 38, 40, 42, 44, 45, 49, 51, 57, 58, 59, 64, 66, 67, 69, 74, 77, 80, 81, 87, 92, 93, 97, 98, 100, 103, 105, 106, 109, 113, 116, 121, 126, 130, 133, 135, 141, 147, 149, 157, 176, 178, 182 and 183 in SEQ ID NO:25. Further HBcAg variants suitable for use in the compositions of the invention, and which may be further modified according to the disclosure of this specification are described in WO 00/198333, WO 00/177158 and WO 00/214478.

As noted above, generally processed HBcAgs (*i.e.*, those which lack leader sequences) will be used in the compositions and vaccine compositions, respectively,

of the invention. The present invention includes vaccine compositions, as well as methods for using these compositions, which employ the above described variant HBcAgs.

Whether the amino acid sequence of a polypeptide has an amino acid sequence
5 that is at least 80%, 85%, 90%, 95%, 97% or 99% identical to one of the above wild-type amino acid sequences, or a subportion thereof, can be determined conventionally using known computer programs such the Bestfit program. When using Bestfit or any other sequence alignment program to determine whether a particular sequence is, for instance, 95% identical to a reference amino acid
10 sequence, the parameters are set such that the percentage of identity is calculated over the full length of the reference amino acid sequence and that gaps in homology of up to 5% of the total number of amino acid residues in the reference sequence are allowed.

The amino acid sequences of the hereinabove mentioned HBcAg variants and
15 precursors are relatively similar to each other. Thus, reference to an amino acid residue of a HBcAg variant located at a position which corresponds to a particular position in SEQ ID NO:25, refers to the amino acid residue which is present at that position in the amino acid sequence shown in SEQ ID NO:25. The homology between these HBcAg variants is for the most part high enough among Hepatitis B
20 viruses that infect mammals so that one skilled in the art would have little difficulty reviewing both the amino acid sequence shown in SEQ ID NO:25 and that of a particular HBcAg variant and identifying "corresponding" amino acid residues. Furthermore, the HBcAg amino acid sequence shown in SEQ ID NO:24, which shows the amino acid sequence of a HBcAg derived from a virus which infect
25 woodchucks, has enough homology to the HBcAg having the amino acid sequence shown in SEQ ID NO:25 that it is readily apparent that a three amino acid residue insert is present in SEQ ID NO:25 between amino acid residues 155 and 156 of SEQ ID NO:25.

The invention also includes vaccine compositions which comprise HBcAg
30 variants of Hepatitis B viruses which infect birds, as wells as vaccine compositions which comprise fragments of these HBcAg variants. For these HBcAg variants one, two, three or more of the cysteine residues naturally present in these polypeptides

could be either substituted with another amino acid residue or deleted prior to their inclusion in vaccine compositions of the invention.

As discussed above, the elimination of free cysteine residues reduces the number of sites where toxic components can bind to the HBcAg, and also eliminates
5 sites where cross-linking of lysine and cysteine residues of the same or of neighboring HBcAg molecules can occur. Therefore, in another embodiment of the present invention, one or more cysteine residues of the Hepatitis B virus capsid protein have been either deleted or substituted with another amino acid residue.

In other embodiments, compositions and vaccine compositions, respectively, of
10 the invention will contain HBcAgs from which the C-terminal region (*e.g.*, amino acid residues 145-185 or 150-185 of SEQ ID NO: 25) has been removed. Thus, additional modified HBcAgs suitable for use in the practice of the present invention include C-terminal truncation mutants. Suitable truncation mutants include HBcAgs where 1, 5, 10, 15, 20, 25, 30, 34, 35, amino acids have been removed from the
15 C-terminus.

HBcAgs suitable for use in the practice of the present invention also include N-terminal truncation mutants. Suitable truncation mutants include modified HBcAgs where 1, 2, 5, 7, 9, 10, 12, 14, 15, or 17 amino acids have been removed from the N-terminus.

20 Further HBcAgs suitable for use in the practice of the present invention include N- and C-terminal truncation mutants. Suitable truncation mutants include HBcAgs where 1, 2, 5, 7, 9, 10, 12, 14, 15, and 17 amino acids have been removed from the N-terminus and 1, 5, 10, 15, 20, 25, 30, 34, 35 amino acids have been removed from the C-terminus.

25 The invention further includes compositions and vaccine compositions, respectively, comprising HBcAg polypeptides comprising, or alternatively essentially consisting of, or alternatively consisting of, amino acid sequences which are at least 80%, 85%, 90%, 95%, 97%, or 99% identical to the above described truncation mutants.

30 In certain embodiments of the invention, a lysine residue is introduced into a HBcAg polypeptide, to mediate the binding of the A β 1-6 peptide to the VLP of HBcAg. In preferred embodiments, compositions of the invention are prepared using

a HBcAg comprising, or alternatively consisting of, amino acids 1-144, or 1-149, 1-185 of SEQ ID NO:25, which is modified so that the amino acids corresponding to positions 79 and 80 are replaced with a peptide having the amino acid sequence of Gly-Gly-Lys-Gly-Gly (SEQ ID NO:33) resulting in the HBcAg polypeptide having the sequence shown in SEQ ID NO: 26. These compositions are particularly useful in those embodiments where an antigenic determinant is coupled to a VLP of HBcAg. In further preferred embodiments, the cysteine residues at positions 48 and 107 of SEQ ID NO:25 are mutated to serine. The invention further includes compositions comprising the corresponding polypeptides having amino acid sequences shown in any of the hereinabove mentioned Hepatitis B core antigen precursor variants which also have above noted amino acid alterations. Further included within the scope of the invention are additional HBcAg variants which are capable of associating to form a capsid or VLP and have the above noted amino acid alterations. Thus, the invention further includes compositions and vaccine compositions, respectively, comprising HBcAg polypeptides which comprise, or alternatively consist of, amino acid sequences which are at least 80%, 85%, 90%, 95%, 97% or 99% identical to any of the wild-type amino acid sequences, and forms of these proteins which have been processed, where appropriate, to remove the N-terminal leader sequence and modified with above noted alterations.

Compositions or vaccine compositions of the invention may comprise mixtures of different HBcAgs. Thus, these vaccine compositions may be composed of HBcAgs which differ in amino acid sequence. For example, vaccine compositions could be prepared comprising a "wild-type" HBcAg and a modified HBcAg in which one or more amino acid residues have been altered (*e.g.*, deleted, inserted or substituted). Further, preferred vaccine compositions of the invention are those which present highly ordered and repetitive antigen arrays, wherein the antigen is a A β 1-6 peptide.

In a further preferred embodiment of the present invention, the at least one A β 1-6 peptide is bound to said virus-like particle and core particle, respectively, by at least one covalent bond. Preferably, the least one A β 1-6 peptide is bound to the virus-like particle and core particle, respectively, by at least one covalent bond, said covalent bond being a non-peptide bond leading to a A β 1-6 peptide array and A β 1-6

peptide -VLP conjugate, respectively. This A β 1-6 peptide array and conjugate, respectively, has typically and preferably a repetitive and ordered structure since the at least one A β 1-6 peptide is bound to the VLP and core particle, respectively, in an oriented manner. The formation of a repetitive and ordered A β 1-6 peptide -VLP array and conjugate, respectively, is ensured by an oriented and directed as well as defined binding and attachment, respectively, of the at least one A β 1-6 peptide to the VLP and core particle, respectively, as will become apparent in the following. Furthermore, the typical inherent highly repetitive and organized structure of the VLPs and core particles, respectively, advantageously contributes to the display of the A β 1-6 peptide in a highly ordered and repetitive fashion leading to a highly organized and repetitive A β 1-6 peptide -VLP array and conjugate, respectively.

Therefore, the preferred inventive conjugates and arrays, respectively, differ from prior art conjugates in their highly organized structure, dimensions, and in the repetitiveness of the antigen on the surface of the array. The preferred embodiment of this invention, furthermore, allows expression of the particle in an expression host guaranteeing proper folding and assembly of the VLP, to which the antigen, i.e. the A β 1-6 peptide, is then further coupled

The present invention discloses methods of binding of A β 1-6 peptide to VLPs. As indicated, in one aspect of the invention, the A β 1-6 peptide is bound to the VLP by way of chemical cross-linking, typically and preferably by using a heterobifunctional cross-linker. Several hetero-bifunctional cross-linkers are known to the art. In preferred embodiments, the hetero-bifunctional cross-linker contains a functional group which can react with preferred first attachment sites, i.e. with the side-chain amino group of lysine residues of the VLP or at least one VLP subunit, and a further functional group which can react with a preferred second attachment site, i.e. a cysteine residue fused to the A β 1-6 peptide and optionally also made available for reaction by reduction. The first step of the procedure, typically called the derivatization, is the reaction of the VLP with the cross-linker. The product of this reaction is an activated VLP, also called activated carrier. In the second step, unreacted cross-linker is removed using usual methods such as gel filtration or dialysis. In the third step, the A β 1-6 peptide is reacted with the activated VLP, and

this step is typically called the coupling step. Unreacted A β 1-6 peptide may be optionally removed in a fourth step, for example by dialysis. Several hetero-bifunctional cross-linkers are known to the art. These include the preferred cross-linkers SMPH (Pierce), Sulfo-MBS, Sulfo-EMCS, Sulfo-GMBS, Sulfo-SIAB, Sulfo-SMPB, Sulfo-SMCC, SVSB, SIA and other cross-linkers available for example from the Pierce Chemical Company (Rockford, IL, USA), and having one functional group reactive towards amino groups and one functional group reactive towards cysteine residues. The above mentioned cross-linkers all lead to formation of a thioether linkage. Another class of cross-linkers suitable in the practice of the invention is characterized by the introduction of a disulfide linkage between the A β 1-6 peptide and the VLP upon coupling. Preferred cross-linkers belonging to this class include for example SPDP and Sulfo-LC-SPDP (Pierce). The extent of derivatization of the VLP with cross-linker can be influenced by varying experimental conditions such as the concentration of each of the reaction partners, the excess of one reagent over the other, the pH, the temperature and the ionic strength. The degree of coupling, i.e. the amount of A β 1-6 peptides per subunits of the VLP can be adjusted by varying the experimental conditions described above to match the requirements of the vaccine.

A particularly favored method of binding of A β 1-6 peptides to the VLP, is the linking of a lysine residue on the surface of the VLP with a cysteine residue on the A β 1-6 peptide. In some embodiments, fusion of an amino acid linker containing a cysteine residue, as a second attachment site or as a part thereof, to A β 1-6 for coupling to the VLP may be required.

In general, flexible amino acid linkers are favored. Examples of the amino acid linker are selected from the group consisting of: (a) CGG; (b) N-terminal gamma 1-linker; (c) N-terminal gamma 3-linker; (d) Ig hinge regions; (e) N-terminal glycine linkers; (f) (G)_kC(G)_n with n=0-12 and k=0-5 (SEQ ID NO: 34); (g) N-terminal glycine-serine linkers; (h) (G)_kC(G)_m(S)_l(GGGGS)_n with n=0-3, k=0-5, m=0-10, l=0-2 (SEQ ID NO: 35); (i) GGC; (k) GGC-NH₂; (l) C-terminal gamma 1-linker; (m) C-terminal gamma 3-linker; (n) C-terminal glycine linkers; (o) (G)_nC(G)_k with n=0-12 and k=0-5 (SEQ ID NO: 36); (p) C-terminal glycine-serine linkers; (q)

(G)_m(S)_l(GGGGS)_n(G)_oC(G)_k with n=0-3, k=0-5, m=0-10, l=0-2, and o=0-8 (SEQ ID NO: 37).

Further examples of amino acid linkers are the hinge region of Immunoglobulins, glycine serine linkers (GGGGS)_n (SEQ ID NO: 38), and glycine
 5 linkers (G)_n all further containing a cysteine residue as second attachment site and optionally further glycine residues. Typically preferred examples of said amino acid linkers are N-terminal gamma1: CGDKTHTSPP (SEQ ID NO: 39); C-terminal
 gamma 1: DKTHTSPPCG (SEQ ID NO: 40); N-terminal gamma 3: CGGPKPSTPPGSSGGAP (SEQ ID NO: 41); C-terminal gamma 3:
 10 PKPSTPPGSSGGAPGGCG (SEQ ID NO: 42); N-terminal glycine linker: GCGGGG (SEQ ID NO: 43) and C-terminal glycine linker: GGGGCG (SEQ ID NO: 44).

Other amino acid linkers particularly suitable in the practice of the invention, when a hydrophobic Aβ peptide is bound to a VLP, are CGKKGG (SEQ ID NO: 46),
 15 or CGDEGG (SEQ ID NO: 31) for N-terminal linkers, or GGKKGC (SEQ ID NO: 45) and GGEDGC (SEQ ID NO: 32), for the C-terminal linkers. For the C-terminal linkers, the terminal cysteine is optionally C-terminally amidated.

In preferred embodiments of the present invention, GGCG (SEQ ID NO: 47), GGC or GGC-NH₂ ("NH₂" stands for amidation) linkers at the C-terminus of the
 20 peptide or CGG at its N-terminus are preferred as amino acid linkers. In general, glycine residues will be inserted between bulky amino acids and the cysteine to be used as second attachment site, to avoid potential steric hindrance of the bulkier amino acid in the coupling reaction. In the most preferred embodiment of the invention, the amino acid linker GGC-NH₂ is fused to the C-terminus of Aβ1-6.

25 The cysteine residue present on the Aβ1-6 peptide has to be in its reduced state to react with the hetero-bifunctional cross-linker on the activated VLP, that is a free cysteine or a cysteine residue with a free sulfhydryl group has to be available. In the instance where the cysteine residue to function as binding site is in an oxidized form, for example if it is forming a disulfide bridge, reduction of this disulfide bridge with
 30 e.g. DTT, TCEP or β-mercaptoethanol is required. Low concentrations of reducing agent are compatible with coupling as described in WO 02/056905, higher concentrations inhibit the coupling reaction, as a skilled artisan would know, in

which case the reductant has to be removed or its concentration decreased prior to coupling, *e.g.* by dialysis, gel filtration or reverse phase HPLC.

Binding of the A β 1-6 peptide to the VLP by using a hetero-bifunctional cross-linker according to the preferred methods described above, allows coupling of A β 1-6 peptide to the VLP in an oriented fashion. Other methods of binding the A β 1-6 peptide to the VLP include methods wherein the A β 1-6 peptide is cross-linked to the VLP using the carbodiimide EDC, and NHS. In further methods, the A β 1-6 peptide is attached to the VLP using a homo-bifunctional cross-linker such as glutaraldehyde, DSG, BM[PEO]₄, BS³, (Pierce Chemical Company, Rockford, IL, USA) or other known homo-bifunctional cross-linkers which functional groups reactive towards amine groups or carboxyl groups of the VLP.

Other methods of binding the VLP to a A β 1-6 peptide include methods where the VLP is biotinylated, and the A β 1-6 peptide expressed as a streptavidin-fusion protein, or methods wherein both the A β 1-6 peptide and the VLP are biotinylated, for example as described in WO 00/23955. In this case, the A β 1-6 peptide may be first bound to streptavidin or avidin by adjusting the ratio of A β 1-6 peptide to streptavidin such that free binding sites are still available for binding of the VLP, which is added in the next step. Alternatively, all components may be mixed in a "one pot" reaction. Other ligand-receptor pairs, where a soluble form of the receptor and of the ligand is available, and are capable of being cross-linked to the VLP or the A β 1-6 peptide, may be used as binding agents for binding A β 1-6 peptide to the VLP. Alternatively, either the ligand or the receptor may be fused to the A β 1-6 peptide, and so mediate binding to the VLP chemically bound or fused either to the receptor, or the ligand respectively. Fusion may also be effected by insertion or substitution.

As already indicated, in a favored embodiment of the present invention, the VLP is the VLP of a RNA phage, and in a more preferred embodiment, the VLP is the VLP of RNA phage Q β coat protein.

One or several antigen molecules, *i.e.* a A β 1-6 peptide, can be attached to one subunit of the capsid or VLP of RNA phages coat proteins, preferably through the exposed lysine residues of the VLP of RNA phages, if sterically allowable. A specific feature of the VLP of the coat protein of RNA phages and in particular of the

Q β coat protein VLP is thus the possibility to couple several antigens per subunit. This allows for the generation of a dense antigen array.

In a preferred embodiment of the invention, the binding and attachment, respectively, of the at least one A β 1-6 peptide to the virus-like particle is by way of
5 interaction and association, respectively, between at least one first attachment site of the virus-like particle and at least one second attachment of the antigen or antigenic determinant.

VLPs or capsids of Q β coat protein display a defined number of lysine residues on their surface, with a defined topology with three lysine residues pointing towards
10 the interior of the capsid and interacting with the RNA, and four other lysine residues exposed to the exterior of the capsid. These defined properties favor the attachment of antigens to the exterior of the particle, rather than to the interior of the particle where the lysine residues interact with RNA. VLPs of other RNA phage coat proteins also have a defined number of lysine residues on their surface and a defined
15 topology of these lysine residues.

In further preferred embodiments of the present invention, the first attachment site is a lysine residue and/or the second attachment comprises a sulfhydryl group or a cysteine residue. In a very preferred embodiment of the present invention, the first attachment site is a lysine residue and the second attachment is a cysteine residue.

20 In very preferred embodiments of the invention, the A β 1-6 peptide is bound via a cysteine residue, to lysine residues of the VLP of RNA phage coat protein, and in particular to the VLP of Q β coat protein.

Another advantage of the VLPs derived from RNA phages is their high expression yield in bacteria that allows production of large quantities of material at
25 affordable cost.

As indicated, the inventive conjugates and arrays, respectively, differ from prior art conjugates in their highly organized structure, dimensions, and in the repetitiveness of the antigen on the surface of the array. Moreover, the use of the VLPs as carriers allow the formation of robust antigen arrays and conjugates,
30 respectively, with variable antigen density. In particular, the use of VLPs of RNA phages, and hereby in particular the use of the VLP of RNA phage Q β coat protein allows achieving very high epitope density. In particular, a density of more than 1.5

epitopes per subunit could be reached by coupling the human A β 1-6 peptide to the VLP of Q β coat protein. The preparation of compositions of VLPs of RNA phage coat proteins with a high epitope density can be effected using the teaching of this application. In preferred embodiment of the invention, when a A β 1-6 peptide is
5 coupled to the VLP of Q β coat protein, an average number of A β 1-6 peptide per subunit of 0.5, 0.6, 0.7, 0.8, 0.9, 1.0, 1.1, 1.2, 1.3, 1.4, 1.5, 1.6, 1.7, 1.8, 1.9, 2.0, 2.1, 2.2, 2.3, 2.4, 2.5, 2.6, 2.7, 2.8, 2.9, or higher is preferred.

The second attachment site, as defined herein, may be either naturally or non-naturally present with the antigen or the antigenic determinant. In the case of the
10 absence of a suitable natural occurring second attachment site on the antigen or antigenic determinant, such a, then non-natural second attachment has to be engineered to the antigen.

As described above, four lysine residues are exposed on the surface of the VLP of Q β coat protein. Typically these residues are derivatized upon reaction with a
15 cross-linker molecule. In the instance where not all of the exposed lysine residues can be coupled to an antigen, the lysine residues which have reacted with the cross-linker are left with a cross-linker molecule attached to the ϵ -amino group after the derivatization step. This leads to disappearance of one or several positive charges, which may be detrimental to the solubility and stability of the VLP. By replacing
20 some of the lysine residues with arginines, as in the disclosed Q β coat protein mutants described below, we prevent the excessive disappearance of positive charges since the arginine residues do not react with the cross-linker. Moreover, replacement of lysine residues by arginines may lead to more defined antigen arrays, as fewer sites are available for reaction to the antigen.

25 Accordingly, exposed lysine residues were replaced by arginines in the following Q β coat protein mutants and mutant Q β VLPs disclosed in this application: Q β -240 (Lys13-Arg; SEQ ID NO:17), Q β -250 (Lys 2-Arg, Lys13-Arg; SEQ ID NO: 19) and Q β -259 (Lys 2-Arg, Lys16-Arg; SEQ ID NO:21). The constructs were cloned, the proteins expressed, the VLPs purified and used for coupling to peptide
30 and protein antigens. Q β -251 ; (SEQ ID NO: 20 was also constructed, and guidance

on how to express, purify and couple the VLP of Q β -251 coat protein can be found throughout the application.

In a further embodiment, we disclose a Q β mutant coat protein with one additional lysine residue, suitable for obtaining even higher density arrays of
5 antigens. This mutant Q β coat protein, Q β -243 (Asn 10-Lys; SEQ ID NO: 18), was cloned, the protein expressed, and the capsid or VLP isolated and purified, showing that introduction of the additional lysine residue is compatible with self-assembly of the subunits to a capsid or VLP. Thus, A β 1-6 peptide arrays and conjugates, respectively, may be prepared using VLP of Q β coat protein mutants. A particularly
10 favored method of attachment of antigens to VLPs, and in particular to VLPs of RNA phage coat proteins is the linking of a lysine residue present on the surface of the VLP of RNA phage coat proteins with a cysteine residue added to the antigen, i.e. the A β 1-6 peptide. In order for a cysteine residue to be effective as second attachment site, a sulfhydryl group must be available for coupling. Thus, a cysteine
15 residue has to be in its reduced state, that is, a free cysteine or a cysteine residue with a free sulfhydryl group has to be available. In the instant where the cysteine residue to function as second attachment site is in an oxidized form, for example if it is forming a disulfide bridge, reduction of this disulfide bridge with *e.g.* DTT, TCEP or β -mercaptoethanol is required. The concentration of reductant, and the molar excess
20 of reductant over antigen has to be adjusted for each antigen. A titration range, starting from concentrations as low as 10 μ M or lower, up to 10 to 20 mM or higher reductant if required is tested, and coupling of the antigen to the carrier assessed. Although low concentrations of reductant are compatible with the coupling reaction as described in WO 02/056905, higher concentrations inhibit the coupling reaction,
25 as a skilled artisan would know, in which case the reductant has to be removed or its concentration decreased, *e.g.* by dialysis, gel filtration or reverse phase HPLC . Advantageously, the pH of the dialysis or equilibration buffer is lower than 7, preferably 6. The compatibility of the low pH buffer with antigen activity or stability has to be tested.

30 Epitope density on the VLP of RNA phage coat proteins can be modulated by the choice of cross-linker and other reaction conditions. For example, the cross-linkers Sulfo-GMBS and SMPH typically allow reaching high epitope density.

Derivatization is positively influenced by high concentration of reactands, and manipulation of the reaction conditions can be used to control the number of antigens coupled to VLPs of RNA phage coat proteins, and in particular to VLPs of Q β coat protein.

5 Prior to the design of a non-natural second attachment site the position at which it should be fused, inserted or generally engineered has to be chosen. The selection of the position of the second attachment site may, by way of example, be based on a crystal structure of the antigen. Such a crystal structure of the antigen may provide information on the availability of the C- or N-termini of the molecule
10 (determined for example from their accessibility to solvent), or on the exposure to solvent of residues suitable for use as second attachment sites, such as cysteine residues. Exposed disulfide bridges, as is the case for Fab fragments, may also be a source of a second attachment site, since they can be generally converted to single cysteine residues through mild reduction, with e.g. 2-mercaptoethylamine, TCEP, β -
15 mercaptoethanol or DTT. Mild reduction conditions not affecting the immunogenicity of the antigen will be chosen. In general, in the case where immunization with a self-antigen is aiming at inhibiting the interaction of this self-antigen with its natural ligands, the second attachment site will be added such that it allows generation of antibodies against the site of interaction with the natural ligands.
20 Thus, the location of the second attachment site will be selected such that steric hindrance from the second attachment site or any amino acid linker containing the same is avoided. In further embodiments, an antibody response directed at a site distinct from the interaction site of the self-antigen with its natural ligand is desired. In such embodiments, the second attachment site may be selected such that it
25 prevents generation of antibodies against the interaction site of the self-antigen with its natural ligands.

Other criteria in selecting the position of the second attachment site include the oligomerization state of the antigen, the site of oligomerization, the presence of a cofactor, and the availability of experimental evidence disclosing sites in the antigen
30 structure and sequence where modification of the antigen is compatible with the function of the self-antigen, or with the generation of antibodies recognizing the self-antigen.

In the most preferred embodiments, the A β 1-6 peptide comprises a single second attachment site or a single reactive attachment site capable of association with the first attachment sites on the core particle and the VLPs or VLP subunits, respectively. This ensures a defined and uniform binding and association, respectively, of the at least one, but typically more than one, preferably more than 10, 20, 40, 80, 120, 150, 180, 210, 240, 270, 300, 360, 400, 450 antigens to the core particle and VLP, respectively. The provision of a single second attachment site or a single reactive attachment site on the antigen, thus, ensures a single and uniform type of binding and association, respectively leading to a very highly ordered and repetitive array. For example, if the binding and association, respectively, is effected by way of a lysine- (as the first attachment site) and cysteine- (as a second attachment site) interaction, it is ensured, in accordance with this preferred embodiment of the invention, that only one cysteine residue per antigen, independent whether this cysteine residue is naturally or non-naturally present on the antigen, is capable of binding and associating, respectively, with the VLP and the first attachment site of the core particle, respectively.

In some embodiments, engineering of a second attachment site onto the antigen require the fusion of an amino acid linker containing an amino acid suitable as second attachment site according to the disclosures of this invention. Therefore, in a preferred embodiment of the present invention, an amino acid linker is bound to the antigen or the antigenic determinant by way of at least one covalent bond. Preferably, the amino acid linker comprises, or alternatively consists of, the second attachment site. In a further preferred embodiment, the amino acid linker comprises a sulfhydryl group or a cysteine residue. In another preferred embodiment, the amino acid linker is cysteine. Some criteria of selection of the amino acid linker as well as further preferred embodiments of the amino acid linker according to the invention have already been mentioned above.

In a further preferred embodiment of the invention, the at least one antigen or antigenic determinant, i.e. the A β 1-6 peptide is fused to the virus-like particle. As outlined above, a VLP is typically composed of at least one subunit assembling into a VLP. Thus, in again a further preferred embodiment of the invention, the antigen or antigenic determinant, preferably the at least one A β 1-6 peptide is fused to at least

one subunit of the virus-like particle or of a protein capable of being incorporated into a VLP generating a chimeric VLP-subunit- A β 1-6 peptide protein fusion.

Fusion of the A β 1-6 peptides can be effected by insertion into the VLP subunit sequence, or by fusion to either the N- or C-terminus of the VLP-subunit or protein
5 capable of being incorporated into a VLP. Hereinafter, when referring to fusion proteins of a peptide to a VLP subunit, the fusion to either ends of the subunit sequence or internal insertion of the peptide within the subunit sequence are encompassed.

Fusion may also be effected by inserting the A β 1-6 peptide sequences into a
10 variant of a VLP subunit where part of the subunit sequence has been deleted, that are further referred to as truncation mutants. Truncation mutants may have N- or C-terminal, or internal deletions of part of the sequence of the VLP subunit. For example, the specific VLP HBcAg with, for example, deletion of amino acid residues 79 to 81 is a truncation mutant with an internal deletion. Fusion of A β 1-6 peptides to
15 either the N- or C-terminus of the truncation mutants VLP-subunits also lead to embodiments of the invention. Likewise, fusion of an epitope into the sequence of the VLP subunit may also be effected by substitution, where for example for the specific VLP HBcAg, amino acids 79-81 are replaced with a foreign epitope. Thus, fusion, as referred to hereinafter, may be effected by insertion of the A β 1-6 peptide
20 sequence in the sequence of a VLP subunit, by substitution of part of the sequence of the VLP subunit with the A β 1-6 peptide, or by a combination of deletion, substitution or insertions.

The chimeric A β 1-6 peptide-VLP subunit will be in general capable of self-assembly into a VLP. VLP displaying epitopes fused to their subunits are also herein
25 referred to as chimeric VLPs. As indicated, the virus-like particle comprises or alternatively is composed of at least one VLP subunit. In a further embodiment of the invention, the virus-like particle comprises or alternatively is composed of a mixture of chimeric VLP subunits and non-chimeric VLP subunits, i.e. VLP subunits not having an antigen fused thereto, leading to so called mosaic particles. This may be
30 advantageous to ensure formation of, and assembly to a VLP. In those embodiments, the proportion of chimeric VLP-subunits may be 1, 2, 5, 10, 20, 30, 40, 50, 60, 70, 80, 90, 95% or higher.

Flanking amino acid residues may be added to either end of the sequence of the peptide or epitope to be fused to either end of the sequence of the subunit of a VLP, or for internal insertion of such peptidic sequence into the sequence of the subunit of a VLP. Glycine and serine residues are particularly favored amino acids to be used in the flanking sequences added to the peptide to be fused. Glycine residues confer additional flexibility, which may diminish the potentially destabilizing effect of fusing a foreign sequence into the sequence of a VLP subunit.

In a specific embodiment of the invention, the VLP is a Hepatitis B core antigen VLP. Fusion proteins of the A β 1-6 peptide to either the N-terminus of a HBcAg (Neyrinck, S. *et al.*, *Nature Med.* 5:1157-1163 (1999)) or insertions in the so called major immunodominant region (MIR) have been described (Pumpens, P. and Grens, E., *Intervirology* 44:98-114 (2001)), WO 01/98333), and are preferred embodiments of the invention. Naturally occurring variants of HBcAg with deletions in the MIR have also been described (Pumpens, P. and Grens, E., *Intervirology* 44:98-114 (2001), which is expressly incorporated by reference in its entirety), and fusions to the N- or C-terminus, as well as insertions at the position of the MIR corresponding to the site of deletion as compared to a wt HBcAg are further embodiments of the invention. Fusions to the C-terminus have also been described (Pumpens, P. and Grens, E., *Intervirology* 44:98-114 (2001)). One skilled in the art will easily find guidance on how to construct fusion proteins using classical molecular biology techniques (Sambrook, J. *et al.*, eds., *Molecular Cloning, A Laboratory Manual*, 2nd. edition, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y. (1989), Ho *et al.*, *Gene* 77:51 (1989)). Vectors and plasmids encoding HBcAg and HBcAg fusion proteins and useful for the expression of a HBcAg and HBcAg fusion proteins have been described (Pumpens, P. & Grens, E. *Intervirology* 44: 98-114 (2001), Neyrinck, S. *et al.*, *Nature Med.* 5:1157-1163 (1999)) and can be used in the practice of the invention. We also describe by way of example (Example 6) the insertion of an epitope into the MIR of HBcAg, resulting in a chimeric self-assembling HBcAg. An important factor for the optimization of the efficiency of self-assembly and of the display of the epitope to be inserted in the MIR of HBcAg is the choice of the insertion site, as well as the number of amino acids to be deleted from the HBcAg sequence within the MIR (Pumpens, P. and Grens, E., *Intervirology*

44:98-114 (2001); EP 0 421 635; U.S. Patent No. 6,231,864) upon insertion, or in other words, which amino acids form HBcAg are to be substituted with the new epitope. For example, substitution of HBcAg amino acids 76-80, 79-81, 79-80, 75-85 or 80-81 with foreign epitopes has been described (Pumpens, P. and Grens, E.,
5 *Intervirology* 44:98-114 (2001); EP0421635; US 6'231'864). HBcAg contains a long arginine tail (Pumpens, P. and Grens, E., *Intervirology* 44:98-114 (2001)) which is dispensable for capsid assembly and capable of binding nucleic acids (Pumpens, P. and Grens, E., *Intervirology* 44:98-114 (2001)). HBcAg either comprising or lacking this arginine tail are both embodiments of the invention.

10 In a further preferred embodiment of the invention, the VLP is a VLP of a RNA phage. The major coat proteins of RNA phages spontaneously assemble into VLPs upon expression in bacteria, and in particular in *E. coli*. Specific examples of bacteriophage coat proteins which can be used to prepare compositions of the invention include the coat proteins of RNA bacteriophages such as bacteriophage Q β
15 (SEQ ID NO:4; PIR Database, Accession No. VCBPQ β referring to Q β CP and SEQ ID NO: 5; Accession No. AAA16663 referring to Q β A1 protein) and bacteriophage ϕ r (SEQ ID NO: 7; PIR Accession No. VCBPFR).

In a more preferred embodiment, the at least one A β 1-6 peptide is fused to a Q β coat protein. Fusion protein constructs wherein epitopes have been fused to the
20 C-terminus of a truncated form of the A1 protein of Q β , or inserted within the A1 protein have been described (Kozlovskaya, T. M., *et al.*, *Intervirology*, 39:9-15 (1996)). The A1 protein is generated by suppression at the UGA stop codon and has a length of 329 aa, or 328 aa, if the cleavage of the N-terminal methionine is taken into account. Cleavage of the N-terminal methionine before an alanine (the second amino
25 acid encoded by the Q β CP gene) usually takes place in *E. coli*, and such is the case for N-termini of the Q β coat proteins. The part of the A1 gene, 3' of the UGA amber codon encodes the CP extension, which has a length of 195 amino acids. Insertion of the at least one A β 1-6 peptide between position 72 and 73 of the CP extension leads to further embodiments of the invention (Kozlovskaya, T. M., *et al.*, *Intervirology* 39:9-
30 15 (1996)). Fusion of a A β 1-6 peptide at the C-terminus of a C-terminally truncated Q β A1 protein leads to further preferred embodiments of the invention. For example,

Kozlovskaya et al., (*Intervirology*, 39: 9-15 (1996)) describe Q β A1 protein fusions where the epitope is fused at the C-terminus of the Q β CP extension truncated at position 19.

As described by Kozlovskaya et al. (*Intervirology*, 39: 9-15 (1996)), assembly of
5 the particles displaying the fused epitopes typically requires the presence of both the A1 protein-A β 1-6 peptide fusion and the wt CP to form a mosaic particle. However, embodiments comprising virus-like particles, and hereby in particular the VLPs of the RNA phage Q β coat protein, which are exclusively composed of VLP subunits having at least one A β 1-6 peptide fused thereto, are also within the scope of the
10 present invention.

The production of mosaic particles may be effected in a number of ways. Kozlovskaya et al., *Intervirology*, 39:9-15 (1996), describe three methods, which all can be used in the practice of the invention. In the first approach, efficient display of the fused epitope on the VLPs is mediated by the expression of the plasmid encoding
15 the Q β A1 protein fusion having a UGA stop codon between CP and CP extension in a E. coli strain harboring a plasmid encoding a cloned UGA suppressor tRNA which leads to translation of the UGA codon into Trp (pISM3001 plasmid (Smiley B.K., et al., *Gene* 134:33-40 (1993))). In another approach, the CP gene stop codon is modified into UAA, and a second plasmid expressing the A1 protein-A β 1-6
20 peptide fusion is cotransformed. The second plasmid encodes a different antibiotic resistance and the origin of replication is compatible with the first plasmid (Kozlovskaya, T. M., et al., *Intervirology* 39:9-15 (1996)). In a third approach, CP and the A1 protein-A β 1-6 peptide fusion are encoded in a bicistronic manner, operatively linked to a promoter such as the Trp promoter, as described in FIG. 1 of Kozlovskaya
25 et al., *Intervirology*, 39:9-15 (1996).

In a further embodiment, the A β 1-6 peptide is inserted between amino acid 2 and 3 (numbering of the cleaved CP, that is wherein the N-terminal methionine is cleaved) of the fr CP, thus leading to a A β 1-6 peptide-fr CP fusion protein. Vectors and expression systems for construction and expression of fr CP fusion proteins self-
30 assembling to VLP and useful in the practice of the invention have been described (Pushko P. et al., *Prot. Eng.* 6:883-891 (1993)). In a specific embodiment, the A β 1-6 peptide sequence is inserted into a deletion variant of the fr CP after amino acid 2,

wherein residues 3 and 4 of the fr CP have been deleted (Pushko P. *et al.*, *Prot. Eng.* 6:883-891 (1993)).

Fusion of epitopes in the N-terminal protuberant β -hairpin of the coat protein of RNA phage MS-2 and subsequent presentation of the fused epitope on the self-assembled VLP of RNA phage MS-2 has also been described (WO 92/13081), and
5 fusion of A β 1-6 peptide by insertion or substitution into the coat protein of MS-2 RNA phage is also falling under the scope of the invention.

In another embodiment of the invention, the A β 1-6 peptide is fused to a capsid protein of papillomavirus. In a more specific embodiment, the A β 1-6 peptide is fused
10 to the major capsid protein L1 of bovine papillomavirus type 1 (BPV-1). Vectors and expression systems for construction and expression of BPV-1 fusion proteins in a baculovirus/insect cells systems have been described (Chackerian, B. *et al.*, *Proc. Natl. Acad. Sci. USA* 96:2373-2378 (1999), WO 00/23955). Substitution of amino acids 130-136 of BPV-1 L1 with a A β 1-6 peptide leads to a BPV-1 L1-A β 1-6
15 peptide fusion protein, which is a preferred embodiment of the invention. Cloning in a baculovirus vector and expression in baculovirus infected Sf9 cells has been described, and can be used in the practice of the invention (Chackerian, B. *et al.*, *Proc. Natl. Acad. Sci. USA* 96:2373-2378 (1999), WO 00/23955). Purification of the assembled particles displaying the fused A β 1-6 peptide can be performed in a
20 number of ways, such as for example gel filtration or sucrose gradient ultracentrifugation (Chackerian, B. *et al.*, *Proc. Natl. Acad. Sci. USA* 96:2373-2378 (1999), WO 00/23955).

In a further embodiment of the invention, the A β 1-6 peptide is fused to a Ty protein capable of being incorporated into a Ty VLP. In a more specific embodiment,
25 the A β 1-6 peptide is fused to the p1 or capsid protein encoded by the TYA gene (Roth, J.F., *Yeast* 16:785-795 (2000)). The yeast retrotransposons Ty1, 2, 3 and 4 have been isolated from *Saccharomyces Serevisiae*, while the retrotransposon Tf1 has been isolated from *Schizosaccharomyces Pombae* (Boeke, J.D. and Sandmeyer, S.B., "Yeast Transposable elements," in *The molecular and Cellular Biology of the*
30 *Yeast Saccharomyces: Genome dynamics, Protein Synthesis, and Energetics*, p. 193, Cold Spring Harbor Laboratory Press (1991)). The retrotransposons Ty1 and 2 are related to the *copia* class of plant and animal elements, while Ty3 belongs to the

gypsy family of retrotransposons, which is related to plants and animal retroviruses. In the Ty1 retrotransposon, the p1 protein, also referred to as Gag or capsid protein, has a length of 440 amino acids. P1 is cleaved during maturation of the VLP at position 408, leading to the p2 protein, the essential component of the VLP.

5 Fusion proteins to p1 and vectors for the expression of said fusion proteins in Yeast have been described (Adams, S.E., *et al.*, *Nature* 329:68-70 (1987)). So, for example, a A β 1-6 peptide may be fused to p1 by inserting a sequence coding for the A β 1-6 peptide into the BamH1 site of the pMA5620 plasmid (Adams, S.E., *et al.*, *Nature* 329:68-70 (1987)). The cloning of sequences coding for foreign epitopes into
10 the pMA5620 vector leads to expression of fusion proteins comprising amino acids 1-381 of p1 of Ty1-15, fused C-terminally to the N-terminus of the foreign epitope. Likewise, N-terminal fusion of a A β 1-6 peptide, or internal insertion into the p1 sequence, or substitution of part of the p1 sequence are also meant to fall within the scope of the invention. In particular, insertion of a A β 1-6 peptide into the Ty
15 sequence between amino acids 30-31, 67-68, 113-114 and 132-133 of the Ty protein p1 (EP0677111) leads to preferred embodiments of the invention.

Further VLPs suitable for fusion of A β 1-6 peptides are, for example, Retrovirus-like-particles (WO9630523), HIV2 Gag (Kang, Y.C., *et al.*, *Biol. Chem.* 380:353-364 (1999)), Cowpea Mosaic Virus (Taylor, K.M.*et al.*, *Biol. Chem.*
20 380:387-392 (1999)), parvovirus VP2 VLP (Rueda, P. *et al.*, *Virology* 263:89-99 (1999)), HBsAg (US 4,722,840, EP0020416B1).

Examples of chimeric VLPs suitable for the practice of the invention are also those described in *Intervirology* 39:1 (1996). Further examples of VLPs contemplated for use in the invention are: HPV-1, HPV-6, HPV-11, HPV-16, HPV-
25 18, HPV-33, HPV-45, CRPV, COPV, HIV GAG, Tobacco Mosaic Virus. Virus-like particles of SV-40, Polyomavirus, Adenovirus, Herpes Simplex Virus, Rotavirus and Norwalk virus have also been made, and chimeric VLPs of those VLPs comprising a A β 1-6 peptide are also within the scope of the present invention.

In preferred embodiments of the invention, A β 1-6 peptides suitable for
30 generating vaccines of the invention are modified with an amino acid linker for binding to a VLP. Those A β 1-6 peptides include, but are not limited to: A β 1-6 fused C-terminally to the linker GGC. Amino acid linkers suitable for fusion to the N-

terminus of A β 1-6 fragments include but are not limited to the sequence CGG and CGHGNKS. Linkers suitable for fusion to the C-terminus of A β 1-6 include but are not limited to the sequence GGC. In a preferred embodiment, when a linker is fused to the C-terminus of A β or A β fragments, the C-terminal Cysteine is amidated. In a preferred embodiment, A β 1-6 is fused to an amino acid linker and has the sequence: “NH₂-DAEFRHGGC-CONH₂, wherein the C-terminal Cysteine is amidated, which is indicated by the C-terminal “-CONH₂”, and the N-terminus of the peptide is free, which is further indicated by “NH₂-“. Amino acid linkers are preferably short, to avoid induction of immune responses against amino acids of said linker, but should allow the induction of antibodies cross-reactive with soluble A β and AD plaques and may facilitate the interaction of antibodies with the A β 1-6 peptide. Other suitable properties of the amino acid linker are flexibility, and preferably lack of bulky amino acids which might interfere with coupling, and/or generate an immune response against the linker itself. In more preferred embodiments, the amino acid linker containing a cysteine residue as second attachment site is fused to the C-terminus of the A β 1-6 peptide.

Additional A β fragments suitable in the practice of the invention include A β fragments corresponding to the aforementioned fragments, also modified as described above, from other animal species and eliciting Antibodies cross-reactive with human amyloid plaques and soluble human A β . Examples of such fragments are A β 1-6 from primates (DAEFRH; SEQ ID NO: 84), rabbit (DAEFRH; SEQ ID NO: 85), guinea pig (DAEFRH; SEQ ID NO: 88), mouse (DAEFGH; SEQ ID NO: 76), rat (DAEFGH SEQ ID NO: 87), and *xaenopus laevis* (DSEYRH; 86).

A number of animal models of AD based on transgenic mice overexpressing mutated forms of human APP have been reported (Games, D. *et al.*, *Nature* 373: 523-527 (1995a); Sturchler-Pierrat *et al.*, *Proc. Natl. Acad. Sci. USA* 94: 13287-13292 (1997); Hsiao, K., *et al.*, *Science* 274: 99-102 (1996); Chen, G. *et al.*, *Nature* 408 : 975- 979 (2000) ; Janus, C. *et al.*, *Nature* 408 : 979-982 (2000) ; Morgan, D. *et al.*, *Nature* 408 : 982-985 (2000)). Those mice models differ from each other in the level of overexpression of the transgene, the AD mutations present on the transgene and the promoter under which overexpression of the transgene is directed. These animal models fail to display all of the pathological signs of AD, which are in

particular age-related changes in behaviour, deposition of β -amyloid into insoluble plaques, neurofibrillary tangles within neurons, and loss of neurons throughout the forebrain (Chapman, P.F. *Nature* 408: 915-916 (2000)). Memory deficits and methods to identify them could however be identified in those models, and may be
5 used in testing the effect of the compositions of the invention in animal models (Chen, G. *et al.*, *Nature* 408 : 975- 979 (2000) ; Janus, C. *et al.*, *Nature* 408 : 979-982 (2000) ; Morgan, D. *et al.*, *Nature* 408 : 982-985 (2000)). Furthermore, age related deposition of A β into amyloid plaques can be studied in those models, which also develop astrogliosis and microgliosis.

10 It will be understood by one of ordinary skill in the relevant arts that other suitable modifications and adaptations to the methods and applications described herein are readily apparent and may be made without departing from the scope of the invention or any embodiment thereof. Having now described the present invention in detail, the same will be more clearly understood by reference to the following
15 examples, which are included herewith for purposes of illustration only and are not intended to be limiting of the invention.

EXAMPLES

EXAMPLE 1

20 **Cloning and construction, respectively, expression and purification of preferred core particles and VLP of RNA phages, respectively**

- A. Construction and expression of mutant Q β coat proteins, and purification of mutant Q β coat protein VLPs or Capsids.

Plasmid construction and cloning of mutant coat proteins

Construction of pQ β -240:

25 The plasmid pQ β 10 (Kozlovskaya, TM, *et al.*, *Gene* 137:133-137) was used as an initial plasmid for the construction of pQ β -240. The mutation Lys13 \rightarrow Arg was created by inverse PCR. The inverse primers were designed in inverted tail-to-tail directions:

5'-GGTAACATCGGT**TC**GAGATGGAAAACAACTCTGGTCC-3'
(SEQ ID NO: 48)

and

5'-GGACCAGAGTTTGT**TTT**CCATCTCGACCGATGTTACC-3'
5 (SEQ ID NO: 49).

The products of the first PCR were used as templates for the second PCR reaction, in which an upstream primer

5'-AGCTCGCCCGGGGATCCTCTAG-3' (SEQ ID NO: 50)

and a downstream primer

10 5'-CGATGCATTTTCATCCTTAGTTATCAATACGCTGGGTTCAG-
3' (SEQ ID NO: 51)

were used. The product of the second PCR was digested with *XbaI* and *MphI103I* and cloned into the pQ β 10 expression vector, which was cleaved by the same restriction enzymes. The PCR reactions were performed with
15 PCR kit reagents and according to producer protocol (MBI Fermentas, Vilnius, Lithuania).

Sequencing using the direct label incorporation method verified the desired mutations. *E.coli* cells harbouring pQ β -240 supported efficient synthesis of 14-kD protein co migrating upon SDS-PAGE with control Q β coat protein isolated from Q β
20 phage particles.

Resulting amino acid sequence: (SEQ ID NO: 17)

AKLETVTLGNIG**RD**GKQTLVLNPRGVNPTNGVASLSQAGAVP
ALEKRVTVSVSQPSRNRKNYKVQVKIQNPTACTANGSCDPSVTRQ
KYADVTFSTQYSTDEERAFVRTELAALLASPLLIDAIDQLNPAY

25

Construction of pQ β -243:

The plasmid pQ β 10 was used as an initial plasmid for the construction of pQ β -243. The mutation Asn10 \rightarrow Lys was created by inverse PCR. The inverse primers were designed in inverted tail-to-tail directions:

30 5'-GGCAA**A**ATTAGAGACTGTTACTTTAGGTAAGATCGG -3'
(SEQ ID NO: 52)

and

5'-CCGATCTTACCTAAAGTAACAGTCTCTAATTTTGCC -3'

(SEQ ID NO: 53).

The products of the first PCR were used as templates for the second PCR
5 reaction, in which an upstream primer

5'-AGCTCGCCCCGGGGATCCTCTAG-3' (SEQ ID NO: 50)

and a downstream primer

5'-CGATGCATTTTCATCCTTAGTTATCAATACGCTGGGTTCAG-
3' (SEQ ID NO: 51)

10 were used. The product of the second PCR was digested with *Xba*I
and *Mph*I103I and cloned into the pQ β 10 expression vector, which was cleaved by
the same restriction enzymes. The PCR reactions were performed with PCR kit
reagents and according to producer protocol (MBI Fermentas, Vilnius, Lithuania).

Sequencing using the direct label incorporation method verified the desired
15 mutations. *E.coli* cells harbouring pQ β -243 supported efficient synthesis of 14-kD
protein co migrating upon SDS-PAGE with control Q β coat protein isolated from
Q β phage particles.

Resulting amino acid sequence: (SEQ ID NO: 18)

AKLETVTLGKIGKDQKQTLVLNPRGVNPTNGVASLSQAGAVP
20 ALEKRVTVSVSQPSRNRKNYKVQVKIQNPTACTANGSCDPSVTRQ
KYADVTFSTQYSTDEERAFVRTELAALLASPLLIDAIDQLNPAY

Construction of pQ β -250:

The plasmid pQ β -240 was used as an initial plasmid for the construction of
pQ β -250. The mutation Lys2→Arg was created by site-directed mutagenesis. An
25 upstream primer

5'-GGCCATGGCACGACTCGAGACTGTTACTTTAGG-3' (SEQ
ID NO: 54)

and a downstream primer

5'-GATTTAGGTGACACTATAG-3' (SEQ ID NO: 55)

30 were used for the synthesis of the mutant PCR-fragment, which was introduced
into the pQ β -185 expression vector at the unique restriction sites *Nco*I and *Hind*III.

The PCR reactions were performed with PCR kit reagents and according to producer protocol (MBI Fermentas, Vilnius, Lithuania).

Sequencing using the direct label incorporation method verified the desired mutations. *E.coli* cells harbouring pQ β -250 supported efficient synthesis of 14-kD protein co migrating upon PAGE with control Q β coat protein isolated from Q β phage particles.

Resulting amino acid sequence: (SEQ ID NO: 19)

ARLETVTLGNIGRDGKQTLVLNPRGVNPTNGVASLSQAGAVP

ALEKRVTVSVSQPSRNRKNYKVQVKIQNPTACTANGSCDPSVTRQ

10 KYADVTFSTQYSTDEERAFVRTELAALLASPLLI DAIDQLNPAY

Construction of pQ β -251:

The plasmid pQ β 10 was used as an initial plasmid for the construction of pQ β -251. The mutation Lys16 \rightarrow Arg was created by inverse PCR. The inverse primers were designed in inverted tail-to-tail directions:

15 5'-GATGGACGTCAAACCTCTGGTCCTCAATCCGCGTGGGG -3'
(SEQ ID NO: 56)

and

5'-CCCCACGCGGATTGAGGACCAGAGTTTGACGTCCATC -3'
(SEQ ID NO: 57).

20 The products of the first PCR were used as templates for the second PCR reaction, in which an upstream primer

5'-AGCTCGCCCGGGGATCCTCTAG-3' (SEQ ID NO: 50)

and a downstream primer

25 5'-CGATGCATTTATCCTTAGTTATCAATACGCTGGGTTCAG-
3' (SEQ ID NO: 51)

were used. The product of the second PCR was digested with *Xba*I and *Mph*I103I and cloned into the pQ β 10 expression vector, which was cleaved by the same restriction enzymes. The PCR reactions were performed with PCR kit reagents and according to producer protocol (MBI Fermentas, Vilnius, Lithuania).

30 Sequencing using the direct label incorporation method verified the desired mutations. *E.coli* cells harbouring pQ β -251 supported efficient synthesis of 14-kD

protein co migrating upon SDS-PAGE with control Q β coat protein isolated from Q β phage particles. The resulting amino acid sequence encoded by this construct is shown in (SEQ. ID NO: 20).

5 Construction of pQ β -259:

The plasmid pQ β -251 was used as an initial plasmid for the construction of pQ β -259. The mutation Lys2→Arg was created by site-directed mutagenesis. An upstream primer

10 5'-GGCCATGGCACGACTCGAGACTGTTACTTTAGG-3' (SEQ ID NO: 54)

and a downstream primer

5'-GATTTAGGTGACACTATAG-3' (SEQ ID NO: 55)

were used for the synthesis of the mutant PCR-fragment, which was introduced into the pQ β -185 expression vector at the unique restriction sites *NcoI* and *HindIII*.

15 The PCR reactions were performed with PCR kit reagents and according to producer protocol (MBI Fermentas, Vilnius, Lithuania).

Sequencing using the direct label incorporation method verified the desired mutations. *E.coli* cells harbouring pQ β -259 supported efficient synthesis of 14-kD protein co migrating upon SDS-PAGE with control Q β coat protein isolated from Q β phage particles.

Resulting amino acid sequence: (SEQ ID NO: 21)

AKLETVTLGNIGKDGKQTLVLNPRGVNPTNGVASLSQAGAVP

ALEKRVTVSVSQPSRNRKNYKVQVKIQNPTACTANGSCDPSVTRQ

KYADVTFSTQYSTDEERA FVRTELAALLASPLLID AIDQLNPAY

25 General procedures for Expression and purification of Q β and Q β mutants

Expression

E.coli JM109 was transformed with Q β coat protein expression plasmids. 5 ml of LB liquid medium containing 20 μ g/ml ampicillin were inoculated with clones transformed with with Q β coat protein expression plasmids. The inoculated culture was incubated at 37 °C for 16-24 h without shaking. The prepared inoculum was

subsequently diluted 1:100 in 100-300 ml of fresh LB medium, containing 20 µg/ml ampicillin. and incubated at 37°C overnight without shaking. The resulting second inoculum was diluted 1:50 in M9 medium containing 1 % Casamino acids and 0.2 % glucose in flasks, and incubated at 37 °C overnight under shaking.

5

Purification

Solutions and buffers for the purification procedure:

1. Lysis buffer LB

50mM Tris-HCl pH8,0 with 5mM EDTA , 0,1%

10 tritonX100 and freshly prepared PMSF at a concentration of 5micrograms per ml. Without lysozyme and DNase.

2. SAS

Saturated ammonium sulphate in water

3. Buffer NET.

15 20 mM Tris-HCl, pH 7.8 with 5mM EDTA and 150 mM NaCl.

4. PEG

40% (w/v) polyethylenglycol 6000 in NET

Disruption and lysis

20 Frozen cells were resuspended in LB at 2 ml/g cells. The mixture was sonicated with 22 kH five times for 15 seconds, with intervals of 1min to cool the solution on ice. The lysate was then centrifuged at 14 000 rpm, for 1h using a Janekki K 60 rotor. The centrifugation steps described below were all performed using the same rotor, except otherwise stated. The supernatant was stored at 4° C, while cell
25 debris were washed twice with LB. After centrifugation, the supernatants of the lysate and wash fractions were pooled.

Fractionation

30 A saturated ammonium sulphate solution was added dropwise under stirring to the above pooled lysate. The volume of the SAS was adjusted to be one fifth of total volume, to obtain 20% of saturation. The solution was left standing overnight, and was centrifuged the next day at 14 000 rpm, for 20 min. The pellet was washed with

a small amount of 20% ammonium sulphate, and centrifuged again . The obtained supernatants were pooled, and SAS was added dropwise to obtain 40% of saturation. The solution was left standing overnight, and was centrifuged the next day at 14 000 rpm, for 20 min. The obtained pellet was solubilised in NET buffer.

5

Chromatography

The capsid or VLP protein resolubilized in NET buffer was loaded on a Sepharose CL- 4B column. Three peaks eluted during chromatography. The first one mainly contained membranes and membrane fragments, and was not collected.
10 Capsids were contained in the second peak, while the third one contained other E.coli proteins.

The peak fractions were pooled, and the NaCl concentration was adjusted to a final concentration of 0.65 M. A volume of PEG solution corresponding to one half of the pooled peak fraction was added dropwise under stirring. The solution was left
15 to stand overnight without stirring. The capsid protein was sedimented by centrifugation at 14 000 rpm for 20 min. It was then solubilized in a minimal volume of NET and loaded again on the Sepharose CL- 4B column. The peak fractions were pooled, and precipitated with ammonium sulphate at 60% of saturation (w/v). After centrifugation and resolubilization in NET buffer, capsid protein was loaded on a
20 Sepharose CL-6B column for rechromatography.

Dialysis and drying

The peak fractions obtained above were pooled and extensively dialysed against sterile water, and lyophilized for storage.

25

Expression and purification Q β -240

Cells (*E. coli* JM 109, transformed with the plasmid pQ β -240) were resuspended in LB, sonicated five times for 15 seconds (water ice jacket) and centrifuged at 13000 rpm for one hour. The supernatant was stored at 4°C until
30 further processing, while the debris were washed 2 times with 9 ml of LB, and finally with 9 ml of 0,7 M urea in LB. All supernatants were pooled, and loaded on the Sepharose CL-4B column. The pooled peak fractions were precipitated with

ammonium sulphate and centrifuged. The resolubilized protein was then purified further on a Sepharose 2B column and finally on a Sepharose 6B column. The capsid peak was finally extensively dialyzed against water and lyophilized as described above. The assembly of the coat protein into a capsid was confirmed by electron
5 microscopy.

Expression and purification Q β -243

Cells (*E. coli* RR1) were resuspended in LB and processed as described in the general procedure. The protein was purified by two successive gel filtration steps on
10 the sepharose CL-4B column and finally on a sepharose CL-2B column. Peak fractions were pooled and lyophilized as described above. The assembly of the coat protein into a capsid was confirmed by electron microscopy.

Expression and purification of Q β -250

15 Cells (*E. coli* JM 109, transformed with pQ β -250) were resuspended in LB and processed as described above. The protein was purified by gel filtration on a Sepharose CL-4B and finally on a Sepharose CL-2B column, and lyophilized as described above. The assembly of the coat protein into a capsid was confirmed by electron microscopy.

20

Expression and purification of Q β -259

Cells (*E. coli* JM 109, transformed with pQ β -259) were resuspended in LB and sonicated. The debris were washed once with 10 ml of LB and a second time with 10 ml of 0,7 M urea in LB. The protein was purified by two gel-filtration
25 chromatography steps, on a Sepharose CL-4 B column. The protein was dialyzed and lyophilized, as described above. The assembly of the coat protein into a capsid was confirmed by electron microscopy.

B. Cloning, Expression and Purification of Recombinant AP205 VLP

30

Cloning of the AP205 Coat Protein gene

The cDNA of AP205 coat protein (CP) (SEQ ID NO: 28) was assembled from two cDNA fragments generated from phage AP205 RNA by using a reverse transcription-PCR technique and cloning in the commercial plasmid pCR 4-TOPO for sequencing. Reverse transcription techniques are well known to those of ordinary skill in the relevant art. The first fragment, contained in plasmid p205-246, contained 269 nucleotides upstream of the CP sequence and 74 nucleotides coding for the first 24 N-terminal amino acids of the CP. The second fragment, contained in plasmid p205-262, contained 364 nucleotides coding for amino acids 12-131 of CP and an additional 162 nucleotides downstream of the CP sequence. Both p205-246 and p205-262 were a generous gift from J. Klovins.

The plasmid 283.-58 was designed by two-step PCR, in order to fuse both CP fragments from plasmids p205-246 and p205-262 in one full-length CP sequence.

An upstream primer p1.44 containing the *NcoI* site for cloning into plasmid pQb185, or p1.45 containing the *XbaI* site for cloning into plasmid pQb10, and a downstream primer p1.46 containing the *HindIII* restriction site were used (recognition sequence of the restriction enzyme underlined):

p1.44 5'-AACC ATG GCA AAT AAG CCA ATG CAA CCG-3' (SEQ ID NO: 79)

p1.45 5'-AATCTAGAATTTTCTGCGCACCCATCCCGG-3' (SEQ ID NO: 80)

p1.46 5'-AAAAGC TTA AGC AGT AGT ATC AGA CGA TAC G-3' (SEQ ID NO: 81)

Two additional primers, p1.47, annealing at the 5' end of the fragment contained in p205-262, and p1.48, annealing at the 3' end of the fragment contained in plasmid p205-246 were used to amplify the fragments in the first PCR. Primers p1.47 and p1.48 are complementary to each other.

p1.47: 5'-GAGTGATCCAACCTCGTTTATCAACTACATTT-TCAGCAAGTCTG-3' (SEQ ID NO: 82)

p1.48: 5'-CAGACTTGCTGAAAATGTAGTTGATAAACGA-GTTGGATCACTC-3' (SEQ ID NO: 83)

In the first two PCR reactions, two fragments were generated. The first fragment was generated with primers p1.45 and p1.48 and template p205-246. The

second fragment was generated with primers p1.47 and p1.46, and template p205-262. Both fragments were used as templates for the second PCR reaction, a splice-overlap extension, with the primer combination p1.45 and p1.46 or p1.44 and p1.46. The product of the two second-step PCR reactions were digested with *XbaI* or *NcoI* respectively, and *HindIII*, and cloned with the same restriction sites into pQb10 or pQb185 respectively, two pGEM-derived expression vectors under the control of *E.coli* tryptophan operon promoter.

Two plasmids were obtained, pAP283-58 (SEQ ID NO: 27), containing the gene coding for wt AP205 CP (SEQ ID NO: 28) in pQb10, and pAP281-32 (SEQ ID NO: 30) with mutation Pro5→Thr (SEQ ID NO: 29), in pQb185. The coat protein sequences were verified by DNA sequencing. PAP283-58 contains 49 nucleotides upstream of the ATG codon of the CP, downstream of the *XbaI* site, and contains the putative original ribosomal binding site of the coat protein mRNA.

Expression and Purification of Recombinant AP205 VLP

A. Expression of recombinant AP205 VLP

E.coli JM109 was transformed with plasmid pAP283-58. 5 ml of LB liquid medium with 20 µg/ml ampicillin were inoculated with a single colony, and incubated at 37 °C for 16-24 h without shaking.

The prepared inoculum was diluted 1:100 in 100-300 ml of LB medium, containing 20 µg/ml ampicillin and incubated at 37 °C overnight without shaking. The resulting second inoculum was diluted 1:50 in 2TY medium, containing 0.2 % glucose and phosphate for buffering, and incubated at 37 °C overnight on a shaker. Cells were harvested by centrifugation and frozen at -80°C.

B. Purification of recombinant AP205 VLP

Solutions and buffers:

Lysis buffer

50mM Tris-HCl pH 8.0 with 5mM EDTA , 0.1%

tritonX100 and PMSF at 5 micrograms per
ml.

SAS

Saturated ammonium sulphate in water

5 Buffer NET.

20 mM Tris-HCl, pH 7.8 with 5mM EDTA and
150 mM NaCl.

PEG

40% (w/v) polyethyleneglycol 6000 in NET

10

Lysis:

Frozen cells were resuspended in lysis buffer at 2 ml/g cells. The mixture was
sonicated with 22 kHz five times for 15 seconds, with intervals of 1 min to cool the
solution on ice. The lysate was then centrifuged for 20 minutes at 12 000 rpm, using
15 a F34-6-38 rotor (Eppendorf). The centrifugation steps described below were all
performed using the same rotor, except otherwise stated. The supernatant was stored
at 4° C, while cell debris were washed twice with lysis buffer. After centrifugation,
the supernatants of the lysate and wash fractions were pooled.

Ammonium-sulphate precipitation can be further used to purify AP205 VLP.
20 In a first step, a concentration of ammonium-sulphate at which AP205 VLP does not
precipitate is chosen. The resulting pellet is discarded. In the next step, an
ammonium sulphate concentration at which AP205 VLP quantitatively precipitates is
selected, and AP205 VLP is isolated from the pellet of this precipitation step by
centrifugation (14 000 rpm, for 20 min). The obtained pellet is solubilised in NET
25 buffer.

Chromatography:

The capsid protein from the pooled supernatants was loaded on a Sepharose
4B column (2.8 X 70 cm), and eluted with NET buffer, at 4 ml/hour/fraction.
30 Fractions 28-40 were collected, and precipitated with ammonium sulphate at 60%
saturation. The fractions were analyzed by SDS-PAGE and Western Blot with an
antiserum specific for AP205 prior to precipitation. The pellet isolated by

centrifugation was resolubilized in NET buffer, and loaded on a Sepharose 2B column (2.3 X 65 cm), eluted at 3 ml/h/fraction. Fractions were analysed by SDS-PAGE, and fractions 44-50 were collected, pooled and precipitated with ammonium sulphate at 60% saturation. The pellet isolated by centrifugation was resolubilized in
5 NET buffer, and purified on a Sepharose 6B column (2.5 X 47 cm), eluted at 3 ml/hour/fraction. The fractions were analysed by SDS-PAGE. Fractions 23-27 were collected, the salt concentration adjusted to 0.5 M, and precipitated with PEG 6000, added from a 40% stock in water and to a final concentration of 13.3%. The pellet isolated by centrifugation was resolubilized in NET buffer, and loaded on the same
10 Sepharose 2B column as above, eluted in the same manner. Fractions 43-53 were collected, and precipitated with ammonium sulphate at a saturation of 60%. The pellet isolated by centrifugation was resolubilized in water, and the obtained protein solution was extensively dialyzed against water. About 10 mg of purified protein per gram of cells could be isolated.

15 Examination of the virus-like particles in Electron microscopy showed that they were identical to the phage particles.

EXAMPLE 2

20 Insertion of a peptide containing a Lysine residue into the c/e1 epitope of HBcAg(1-149).

The c/e1 epitope (residues 72 to 88) of HBcAg is located in the tip region on the surface of the Hepatitis B virus capsid (HBcAg). A part of this region (Proline 79 and Alanine 80) was genetically replaced by the peptide Gly-Gly-Lys-Gly-Gly (SEQ ID NO: 33), resulting in the HBcAg-Lys construct (SEQ ID NO: 26). The
25 introduced Lysine residue contains a reactive amino group in its side chain that can be used for intermolecular chemical crosslinking of HBcAg particles with any antigen containing a free cysteine group.

HBcAg-Lys DNA, having the amino acid sequence shown in SEQ ID NO:78, was generated by PCRs: The two fragments encoding HBcAg fragments (amino acid
30 residues 1 to 78 and 81 to 149) were amplified separately by PCR. The primers used for these PCRs also introduced a DNA sequence encoding the Gly-Gly-Lys-Gly-Gly

peptide (SEQ ID NO: 33). The HBcAg (1 to 78) fragment was amplified from pEco63 using primers EcoRIHBcAg(s) and Lys-HBcAg(as). The HBcAg (81 to 149) fragment was amplified from pEco63 using primers Lys-HBcAg(s) and HBcAg(1-149)Hind(as). Primers Lys-HBcAg(as) and Lys-HBcAg(s) introduced
 5 complementary DNA sequences at the ends of the two PCR products allowing fusion of the two PCR products in a subsequent assembly PCR. The assembled fragments were amplified by PCR using primers EcoRIHBcAg(s) and HbcAg(1-149)Hind(as).

For the PCRs, 100 pmol of each oligo and 50 ng of the template DNAs were used in the 50 ml reaction mixtures with 2 units of Pwo polymerase, 0.1 mM dNTPs
 10 and 2 mM MgSO₄. For both reactions, temperature cycling was carried out as follows: 94°C for 2 minutes; 30 cycles of 94°C (1 minute), 50°C (1 minute), 72°C (2 minutes).

Primer sequences:

EcoRIHBcAg(s):

15 (5'-CCGGAATTCATGGACATTGACCCTTATAAAG-3') (SEQ ID NO: 58);

Lys-HBcAg(as):

(5'-CCTAGAGCCACCTTTGCCACCATCTTCTAAATTAG-TACCCACCCAGGTAGC-3') (SEQ ID NO: 59);

Lys-HBcAg(s):

20 (5'-GAAGATGGTGGCAAAGGTGGCTCTAGGGACCTAGTAGTCAGTTATGTC -3') (SEQ ID NO: 60);

HBcAg(1-149)Hind(as):

(5'-CGCGTCCCAAGCTTCTAAACAACAGTAGTCTCCGGAAG-3') (SEQ ID NO: 61).

25 For fusion of the two PCR fragments by PCR 100 pmol of primers EcoRIHBcAg(s) and HBcAg(1-149)Hind(as) were used with 100 ng of the two purified PCR fragments in a 50 ml reaction mixture containing 2 units of Pwo polymerase, 0.1 mM dNTPs and 2 mM MgSO₄. PCR cycling conditions were: 94°C for 2 minutes; 30 cycles of 94°C (1 minute), 50°C (1 minute), 72°C (2 minutes). The
 30 assembled PCR product was analyzed by agarose gel electrophoresis, purified and digested for 19 hours in an appropriate buffer with EcoRI and HindIII restriction

enzymes. The digested DNA fragment was ligated into EcoRI/HindIII-digested pKK vector to generate pKK-HBcAg-Lys expression vector. Insertion of the PCR product into the vector was analyzed by EcoRI/HindIII restriction analysis and DNA sequencing of the insert.

5

EXAMPLE 3

Expression and purification of HBcAg-Lys.

E. coli strains K802 or JM109 were transformed with pKK-HBcAg-Lys. 1 ml of an overnight culture of bacteria was used to inoculate 100 ml of LB medium containing 100 µg/ml ampicillin. This culture was grown for 4 hours at 37°C until
10 an OD at 600 nm of approximately 0.8 was reached. Induction of the synthesis of HBcAg-Lys was performed by addition of IPTG to a final concentration of 1 mM. After induction, bacteria were further shaken at 37°C for 4 hours. Bacteria were harvested by centrifugation at 5000 x g for 15 minutes. The pellet was frozen at -80°C. The pellet was thawed and resuspended in bacteria lysis buffer (10 mM
15 Na₂HPO₄, pH 7.0, 30 mM NaCl, 0.25% Tween-20, 10 mM EDTA) supplemented with 200 µg/ml lysozyme and 10 µl of Benzonase (Merck). Cells were incubated for 30 minutes at room temperature and disrupted by sonication. *E. coli* cells harboring pKK-HBcAg-Lys expression plasmid or a control plasmid were used for induction of HBcAg-Lys expression with IPTG. Prior to the addition of IPTG, a sample was
20 removed from the bacteria culture carrying the pKK-HBcAg-Lys plasmid and from a culture carrying the control plasmid. Four hours after addition of IPTG, samples were again removed from the culture containing pKK-HBcAg-Lys and from the control culture. Protein expression was monitored by SDS-PAGE followed by Coomassie staining.

25 The lysate was then centrifuged for 30 minutes at 12,000 x g in order to remove insoluble cell debris. The supernatant and the pellet were analyzed by Western blotting using a monoclonal antibody against HBcAg (YVS1841, purchased from Accurate Chemical and Scientific Corp., Westbury, NY, USA), indicating that a significant amount of HBcAg-Lys protein was soluble. Briefly, lysates from
30 *E. coli* cells expressing HBcAg-Lys and from control cells were centrifuged at

14,000 x g for 30 minutes. Supernatant (= soluble fraction) and pellet (= insoluble fraction) were separated and diluted with SDS sample buffer to equal volumes. Samples were analyzed by SDS-PAGE followed by Western blotting with anti-HBcAg monoclonal antibody YVS 1841.

5 The cleared cell lysate was used for step-gradient centrifugation using a sucrose step gradient consisting of a 4 ml 65% sucrose solution overlaid with 3 ml 15% sucrose solution followed by 4 ml of bacterial lysate. The sample was centrifuged for 3 hrs with 100,000 x g at 4°C. After centrifugation, 1 ml fractions from the top of the gradient were collected and analyzed by SDS-PAGE followed by
10 Coomassie staining. The HBcAg-Lys protein was detected by Coomassie staining.

 The HBcAg-Lys protein was enriched at the interface between 15 and 65% sucrose indicating that it had formed a capsid particle. Most of the bacterial proteins remained in the sucrose-free upper layer of the gradient, therefore step-gradient centrifugation of the HBcAg-Lys particles led both to enrichment and to a partial
15 purification of the particles.

 Expression and purification of HBcAg-Lys in large scale was performed as follows. An overnight culture was prepared by inoculating a single colony in 100 ml LB, 100 µg/ml Ampicillin and growing the culture overnight at 37°C. 25 ml of the preculture were diluted in 800 ml LB Ampicillin medium the next day, and the
20 culture grown to an optical density OD⁶⁰⁰ of 0.6-0.8. The culture was then induced with 1 mM IPTG, and left to grow for another 4 hours. The cells were harvested and lysed essentially as described above.

 HBcAg-Lys was then purified by first precipitating the protein with ammonium sulphate (30% saturation) from the cleared cell lysate, then loading the resolubilized
25 pellet on a gel filtration column (Sephacryl S-400, Pharmacia). The pooled fractions were precipitated again with ammonium sulphate, the pellet resolubilized and loaded a second time on the same gel filtration column. The fractions were finally pooled and concentrated, and the concentration assessed using a Bradford test (BioRad).

EXAMPLE 4

Construction of a HBcAg devoid of free cysteine residues and containing an inserted lysine residue.

5 A Hepatitis core Antigen (HBcAg), referred to herein as HBcAg-lys-2cys-Mut, devoid of cysteine residues at positions corresponding to 48 and 107 in SEQ ID NO:25 and containing an inserted lysine residue was constructed using the following methods.

The two mutations were introduced by first separately amplifying three fragments of the HBcAg-Lys gene prepared as described above in Example 2 with
10 the following PCR primer combinations. PCR methods and conventional cloning techniques were used to prepare the HBcAg-lys-2cys-Mut gene.

In brief, the following primers were used to prepare fragment 1:

Primer 1: EcoRIHBcAg(s)

CCGGAATTCATGGACATTGACCCTTATAAAG (SEQ ID NO: 58)

15 Primer 2: 48as

GTGCAGTATGGTGAGGTGAGGAATGCTCAGGAGACTC (SEQ ID NO:
62)

The following primers were used to prepare fragment 2:

Primer 3: 48s

20 GSGTCTCCTGAGCATTCCTCACCTCACCATACTGCAC (SEQ ID NO:
63)

Primer 4: 107as

CTTCCAAAAGTGAGGGAAGAAATGTGAAACCAC (SEQ ID NO: 64)

The following primers were used to prepare fragment 3:

25 Primer 5: HBcAg149hind-as

CGCGTCCCAAGCTTCTAAACAACAGTAGTCTCCGGAAGC-
GTTGATAG (SEQ ID NO: 65)

Primer 6: 107s

GTGGTTTCACATTTCTTCCCTCACTTTTGGAAG (SEQ ID NO: 66)

30 Fragments 1 and 2 were then combined with PCR primers EcoRIHBcAg(s) and 107as to give fragment 4. Fragment 4 and fragment 3 were then combined with

primers EcoRIHBcAg(s) and HBcAg149hind-as to produce the full length gene. The full length gene was then digested with the EcoRI (GAATTC) and HindIII (AAGCTT) enzymes and cloned into the pKK vector (Pharmacia) cut at the same restriction sites. Expression and purification of HBcAg-lys-2cys-Mut were performed as set out in Example 3.

EXAMPLE 5

Construction of HBcAg1-185-Lys.

Hepatitis core Antigen (HBcAg) 1-185 was modified as described in Example 2. A part of the c/e1 epitope (residues 72 to 88) region (Proline 79 and Alanine 80) was genetically replaced by the peptide Gly-Gly-Lys-Gly-Gly (SEQ ID NO: 33), resulting in the HBcAg-Lys construct (SEQ ID NO: 26). The introduced Lysine residue contains a reactive amino group in its side chain that can be used for intermolecular chemical crosslinking of HBcAg particles with any antigen containing a free cysteine group. PCR methods and conventional cloning techniques were used to prepare the HBcAg1-185-Lys gene.

The Gly-Gly-Lys-Gly-Gly sequence (SEQ ID NO: 33) was inserted by amplifying two separate fragments of the HBcAg gene from pEco63, as described above in Example 2 and subsequently fusing the two fragments by PCR to assemble the full length gene. The following PCR primer combinations were used:

fragment 1:

Primer 1: EcoRIHBcAg(s) (SEQ ID NO: 58) (see Example 2)

Primer 2: Lys-HBcAg(as) (SEQ ID NO: 59) (see Example 2)

fragment 2:

Primer 3: Lys-HBcAg(s) (SEQ ID NO: 60) (see Example 2)

Primer 4: HBcAgwtHindIII

CGCGTCCCAAGCTTCTAACATTGAGATTCCCGAGATTG (SEQ ID NO: 67)

Assembly:

Primer 1: EcoRIHBcAg(s) (SEQ ID NO: 58) (see example 2)

Primer 2: HBcAgwtHindIII (SEQ ID NO: 67)

The assembled full length gene was then digested with the EcoRI (GAATTC) and HindIII (AAGCTT) enzymes and cloned into the pKK vector (Pharmacia) cut at the same restriction sites.

EXAMPLE 6

5 Fusion of a peptide epitope in the MIR region of HbcAg.

The residues 79 and 80 of HBcAg1-185 were substituted with the epitope CεH3 of sequence VNLTWSRASG (SEQ ID NO: 68). The CεH3 sequence stems from the sequence of the third constant domain of the heavy chain of human IgE. The epitope was inserted in the HBcAg1-185 sequence using an assembly PCR
10 method. In the first PCR step, the HBcAg1-185 gene originating from ATCC clone pEco63 and amplified with primers HBcAg-wt EcoRI fwd and HBcAg-wt Hind III rev was used as template in two separate reactions to amplify two fragments containing sequence elements coding for the CεH3 sequence. These two fragments were then assembled in a second PCR step, in an assembly PCR reaction.

15 Primer combinations in the first PCR step: CεH3fwd with HBcAg-wt Hind III rev, and HBcAg-wt EcoRI fwd with CεH3rev. In the assembly PCR reaction, the two fragments isolated in the first PCR step were first assembled during 3 PCR cycles without outer primers, which were added afterwards to the reaction mixture for the next 25 cycles. Outer primers: HBcAg-wt EcoRI fwd and HBcAg-wt Hind III rev.

20 The PCR product was cloned in the pKK223.3 using the EcoRI and HindIII sites, for expression in *E. coli* (see Example 2). The chimeric VLP was expressed in *E. coli* and purified as described in Example 2. The elution volume at which the HBcAg1-185- CεH3 eluted from the gel filtration showed assembly of the fusion proteins to a chimeric VLP.

Primer sequences:

CεH3fwd:

5' GTT AAC TTG ACC TGG TCT CGT GCT TCT GGT GCA TCC AGG GAT CTA GTA GTC 3'

(SEQ ID NO: 69)

5 V N L T W S R A S G A80 S R D L V V86

(SEQ ID NO: 70)

CεH3rev:

5' ACC AGA AGC ACG AGA CCA GGT CAA GTT AAC ATC TTC CAA ATT ATT ACC CAC 3'

10 (SEQ ID NO: 71)

D78 E L N N G V72

(SEQ ID NO: 72)

HBcAg-wt EcoRI fwd:

5' CCGgaattcATGGACATTGACCCTTATAAAG (SEQ ID NO: 73)

15 HBcAg-wt Hind III rev:

5' CGCGTCCCaaagcttCTAACATTGAGATTCCCGAGATTG (SEQ ID NO:

74)

EXAMPLE 7

Fusion of Aβ1-6 peptide in the MIR region of HbcAg.

20 The residues 79 and 80 of HBcAg1-185 are substituted with the Aβ1-6 peptide of sequence: DAEFRH (SEQ ID NO: 75) or DAEFGH (SEQ ID NO: 76). Two overlapping primers are designed using the same strategy described in Example 6, and the fusion protein constructed by assembly PCR. The PCR product is cloned in the pKK223.3 vector, and expressed in E. coli K802. The chimeric VLPs are

25 expressed and purified as described in Example 3.

EXAMPLE 8

Fusion of a A β 1-6 peptide to the C-terminus of the Q β A1 protein truncated at position 19 of the CP extension.

5 A primer annealing to the 5' end of the Q β A1 gene and a primer annealing to the 3' end of the A1 gene and comprising additionally a sequence element coding for the A β 1-6 peptide, of sequence DAEFRH (SEQ ID NO: 75) or DAEFGH (SEQ ID NO: 76), are used in a PCR reaction with pQ β 10 as template. The PCR product is cloned in pQ β 10 (Kozlovska T.M. *et al.*, *Gene* 137: 133-37 (1993)), and the chimeric VLP expressed and purified as described in Example 1.

10

EXAMPLE 9

Insertion of a A β 1-6 peptide between positions 2 and 3 of fr coat protein.

Complementary primers coding for the sequence of the A β 1-6 peptide of sequence DAEFRH (SEQ ID NO: 75) or DAEFGH (SEQ ID NO: 76), and containing *Bsp*119I compatible ends and additional nucleotides enabling in frame
15 insertion, are inserted in the *Bsp*119I site of the pFrd8 vector (Pushko, P. *et al.*, *Prot. Eng.* 6: 883-91 (1993)) by standard molecular biology techniques. Alternatively, the overhangs of the pFrd8 vector are filled in with Klenow after digestion with *Bsp*119I, and oligonucleotides coding for the sequence of the A β 1-6 peptide and additional nucleotides for in frame cloning are ligated in pFrd8 after the Klenow treatment.
20 Clones with the insert in the right orientation are analysed by sequencing. Expression and purification of the chimeric fusion protein in *E. coli* JM109 or *E. coli* K802 is performed as described in Pushko, P. *et al.*, *Prot. Eng.* 6:883-91 (1993), but for the chromatography steps which are performed using a Sepharose CL-4B or Sephacryl S-400 (Pharmacia) column. The cell lysate is precipitated with ammonium sulphate,
25 and purified by two successive gel filtration purification steps, similarly to the procedure described for Q β in Example 1.

EXAMPLE 10

Insertion of a A β 1-6 peptide between positions 67 and 68 of Ty1 protein p1 in the vector pOGS8111.

Two complementary oligonucleotides coding for the A β 1-6 peptide, of
5 sequence DAEFRH (SEQ ID NO: 75) or DAEFGH (SEQ ID NO: 76), with ends compatible with the NheI site of pOGS8111 are synthesized. Additional nucleotides are added to allow for in frame insertion of a sequence coding for the A β 1-6 peptide according to the description of EP 677'111. The amino acids AS and SS flanking the inserted epitope are encoded by the altered NheI sites resulting from the insertion of
10 the oligonucleotide in the TyA(d) gene of pOGS8111.

POGS8111 is transformed into *S. cerevisiae* strain MC2, for expression of the chimeric Ty VLP as described in EP0677111 and references therein. The chimeric Ty VLP is purified by sucrose gradient ultracentrifugation as described in EP 677'111.

15 EXAMPLE 11

Insertion of a A β 1-6 peptide into the major capsid protein L1 of papillomavirus type 1 (BPV-1).

A sequence coding for the A β 1-6 peptide having the sequence DAEFRH (SEQ ID NO: 75) or DAEFGH (SEQ ID NO: 76) is substituted to the sequence coding for
20 amino acids 130-136 of the BPV-1 L1 gene cloned in the pFastBac1 (GIBCO/BRL) vector as described (Chackerian, B. *et al.*, *Proc. Natl. Acad. USA* 96: 2373-2378 (1999)). The sequence of the construct is verified by nucleotide sequence analysis. Recombinant baculovirus is generated using the GIBCO/BRL baculovirus system as described by the manufacturer. The chimeric VLPs are purified from baculovirus
25 infected Sf9 cells as described by Kirnbauer, R. *et al.*, *Proc. Natl. Acad. Sci.* 89:12180-84 (1992) and Greenstone, H.L., *et al.*, *Proc. Natl. Acad. Sci.* 95:1800-05 (1998).

EXAMPLE 12

Immunization of mice with A β 1-6 peptide fused to VLPs.

Chimeric VLPs displaying the A β 1-6 peptide of sequence DAEFRH (SEQ ID NO: 75) or DAEFGH (SEQ ID NO: 76) generated in Examples 7-11 are used for immunization of human transgenic APP mice or C57/BL6 mice as described in Example 13 and 14. The sera obtained from the immunized mice are analysed in a A β 1-6 peptide or A β 1-40 or A β 1-42 specific ELISA as described in Example 13.

The protective effect of the vaccine is examined by immunizing a large group of human APP transgenic mice as described in Example 14.

10

EXAMPLE 13

Coupling of A β 1-6 peptide to Q β VLP (Q β A β 1-6), and immunization of mice with Q β A β 1-6

A. Coupling of A β 1-6 peptide Q β VLP

The A β 1-6 peptide (sequence: NH₂-DAEFRHGGC-CONH₂) (SEQ ID NO: 77) was chemically synthesized; the initial NH₂ group indicates that the peptide has a free N-terminus, and the terminal NH₂ group indicates that the peptide has an amidated carboxy-terminus. Q β VLP was expressed and purified as described in example 1. Q β VLP, in 20 mM Hepes, 150 mM NaCl, pH 8.2 (HBS, pH 8.2) was reacted at a concentration of 2 mg/ml (determined in a Bradford assay), with 1.43 mM SMPH (Pierce, Rockford IL), diluted from a stock in DMSO, for 30 minutes at room temperature (RT). The reaction mixture was then dialyzed against HBS, pH 8.2 buffer at 4°C, and reacted with 0.36 mM of A β 1-6 peptide, diluted in the reaction mixture from a 50 mM stock in DMSO. The coupling reaction was left to proceed for 2 hours at 15°C, and the reaction mixture dialyzed 2 X 2 hours against a 1000-fold volume HBS, pH 8.2, and flash frozen in liquid nitrogen in aliquots for storage at -80°C until further use.

An aliquot was thawed, and coupling of the A β 1-6 peptide to the Q β VLP subunits assessed by SDS-PAGE and the protein concentration measured in a Bradford assay. The result of the coupling reactions are shown in FIG. 1.

FIG. 1 shows the SDS-PAGE analysis of the coupling reaction of A β 1-6 peptide and Q β VLP. The samples were run under reducing conditions on a 16% Tris-glycine gel, stained with coomassie brilliant blue. Lane 1 is the protein marker, with corresponding molecular weights indicated on the left border of the gel; lane 2, derivatized Q β VLP protein; lane 3, the supernatant of the coupling reaction of Q β VLP protein to the A β 1-6 peptide; lane 4, the pellet of the coupling reaction of Q β VLP protein to the A β 1-6 peptide; Coupling products corresponding to the coupling of 1, 2 and 3 peptides per monomer are indicated by arrows in the Figure. More than 1.5 peptides per subunit were coupled on average; nearly no subunits were left uncoupled.

B. Immunisation of mice with A β 1-6 peptide coupled to Q β VLP and analysis of immune response

Q β VLP coupled to A β 1-6 peptide (denominated here Q β -Ab-1-6) was injected s.c. in mice (3 mice) at day 0 and 14. A β 1-6 peptide was coupled to Q β VLP protein as described above. Each mice (C57BL/6) was immunized with 10 μ g of vaccine diluted in PBS to 200 μ l. Mice were retroorbitally bled on day 21, and the titer of the antibodies specific for the A β 1-6 peptide were measured in an ELISA against A β 1-6. The A β 1-6 peptide was coupled to bovine RNase A using the chemical cross-linker sulfo-SPDP. ELISA plates were coated with coupled RNase preparations at a concentration of 10 μ g/ml. The plates were blocked and then incubated with serially diluted mouse sera. Bound antibodies were detected with enzymatically labeled anti-mouse IgG antibodies. As a control, preimmune sera of the same mice were also tested. The results are shown in FIG. 2.

FIG. 2 shows an ELISA analysis of the IgG antibodies specific for A β 1-6 peptide in sera of mice immunized against the A β 1-6 peptide coupled to Q β VLP. The results are shown for the three mice immunized (A1-A3), the pre-immune serum is indicated as "pre" in the figure; the result for one pre-immune serum is shown.

Comparison of the pre-immune sera with the sera of mice immunized with "Qb-Ab-1-6" shows that a strong specific antibody response against peptide A β 1-6 could be obtained in the absence of adjuvant.

C. ELISA against A β 1-40 peptide

5 Human A β 1-40 or A β 1-42 peptide stock was made in DMSO and diluted in coating buffer before use. ELISA plates were coated with 0.1 μ g /well A β 1-40 peptide. The plates were blocked and then incubated with serially diluted mouse serum obtained above. Bound antibodies were detected with enzymatically labeled anti-mouse IgG antibody. As a control, sera obtained before vaccination were also
10 included. The serum dilution showing a mean three standard deviations above baseline was calculated and defined as "ELISA titer". No specific antibodies were detected in preimmune sera. The titer obtained for the three mice was of 1:100000, showing a strong specific immune response against A β 1-40. Thus, immunization with A β 1-6 coupled to Q β VLP elicits strong antibody titers cross-reactive with
15 A β 1-40.

FIG. 3 shows the result of the ELISA. The ELISA signal as the optical density at 405 nm, obtained for the sera of three mice (A1-A3) immunized with A β 1-6 peptide coupled to Q β VLP as described above, is plotted for each of the dilutions, indicated on the x-axis. The result for the three mice bled at day 21 is shown. Also
20 included is a pre-immune serum. The titer of the antibodies in the sera was determined as described above, and was of 1:100000 for all three mice.

EXAMPLE 14

Immunization of human APP transgenic mice

8 months old female APP23 mice which carry a human APP transgene
25 (Sturchler-Pierrat *et al.*, *Proc.Natl. Acad.Sci. USA* 94: 13287-13292 (1997)) are used for vaccination. The mice are injected subcutaneously with 25 μ g vaccine diluted in sterile PBS and 14 days later boosted with the same amount of vaccine. Mice are bled from the tail vein before the start of immunization and 7 days after the booster

injection. The sera are analyzed for the presence of antibodies specific to a A β 1-6, to A β 1-40 and A β 1-42 by ELISA as described in Example 13.

EXAMPLE 15

5 Coupling of murine A β 1-6 to Q β VLP, injection of the vaccine in mice, and analysis of the immune response

Murine Aβ1-6 peptide (sequence: NH2-DAEFGHGHC-CONH2) (SEQ ID NO: 78) is chemically synthesized, and used for coupling to Qβ VLP as described in Example 13. The vaccine is injected in C57BL/6 mice, and the titer of the elicited antibodies against murine Aβ1-6, murine Aβ1-40 and murine Aβ1-42 determined. The immunization and the ELISA determination are performed as described in Example 13.

EXAMPLE 16

15 Binding of sera elicited against A β 1-6 to human APP transgenic mice plaques and AD plaques

Immunohistochemistry in brain slices

Consecutive paraffin brain sections of a 18 months, old heterozygous APP23 mouse and entorhinal cortex sections from an AD patient Braak Stage III (Institute of Pathology, University Basel) were used for staining. Antigenicity was enhanced by treating human brain sections with concentrated formic acid for five minutes and mouse brain sections by microwave heating at 90 °C for 3 minutes. Mice sera elicited against human A β 1-6 (obtained as described in Example 13) were diluted 1:1000 in PBS with 3% goat serum and incubated over night. Following rinsing, sections were incubated for 1 hour with biotinylated anti mouse secondary antibody diluted 1:200 in PBS. After rinsing, sections were further processed with the avidin-biotin-peroxidase technique (ABC-Elite Kit PK6100; Vector Laboratories). Finally, sections were reacted with Diaminobenzidine (DAB) metal enhanced substrate (Boehringer, Code 1718096), counterstained with Hemalum, dehydrated, cleared in Xylene and coverslipped.

The result of the histologic stains are shown in FIG. 4 A and B. Sections were stained with the sera of the three mice immunized against human A β 1-6 coupled to Q β VLP. Each serum stained positively the amyloid plaques from transgenic mice and AD. Results for one of the three sera are shown. Sera elicited against human A β 1-6 clearly stain amyloid plaques of the transgenic human APP23 mouse, as well as amyloid plaques from AD patients. Pre-immune sera were negative. Extracellular amyloid plaques and isolated blood vessels are stained by the antibodies.

EXAMPLE 17

Specificity of sera elicited against human A β 1-6, assessed by histology of mice plaques

Immunohistochemistry in brain slices

Consecutive paraffin brain sections of a 3 months and an 18 months old heterozygous APP23 mouse overexpressing human APP were stained as described in Example 16 with a representative mouse serum elicited against human A β 1-6 as described in Example 13, or with a rabbit polyclonal antibody specific for the last 20 amino acids of murine or human APP and which therefore does not recognizes A β . The sections incubated with the rabbit polyclonal antibody were treated as described in Example 16, except for the use of a biotinylated anti rabbit secondary antibody (BA1000, Vector Laboratories).

The result of the histologic stains are shown on FIG. 5 A, B, C, D and E. A β 1-6, marked on the bottom left of the sections indicate that sera elicited against A β 1-6 have been used for the staining, while "Pab" indicates that the sections have been stained with the polyclonal antibody specific for the last 20 amino acids of murine or human APP, corresponding to positions 676-695 in APP₆₉₅.

Comparison of the staining of sections from 18 months old mice (FIG. 5 A and C) shows that the sera elicited against A β 1-6 do not cross-react with APP expressed in the brain, which is however stained by the control polyclonal antibody. FIG. 5 B shows a brain section from a 3 months old mouse, a timepoint where amyloid deposits are not yet visible, stained with the polyclonal antibody specific for APP.

FIG. 5 D and 5E show a magnification of the CA1 pyramidal layer of the hippocampus from FIG. 5A. and FIG. 5B, respectively.

EXAMPLE 18

A. Coupling of A β 1-6 peptide to fr capsid protein

5 A solution of 120 μ M fr capsid protein in 20 mM Hepes, 150 mM NaCl pH 7.2 is reacted for 30 minutes with a 10 fold molar excess of SMPH (Pierce), diluted from a stock solution in DMSO, at 25 °C on a rocking shaker. The reaction solution is subsequently dialyzed twice for 2 hours against 1 L of 20 mM Hepes, 150 mM NaCl, pH 7.2 at 4 °C. The dialyzed fr reaction mixture is then reacted with a a five-fold
10 molar excess of A β 1-6 peptide (sequence: NH₂-DAEFRHGGC-CONH₂) (SEQ ID NO: 77) for 2 hours at 16 °C on a rocking shaker. Coupling products are analysed by SDS-PAGE.

B. Coupling of A β 1-6 peptide to HBcAg-Lys-2cys-Mut

A solution of 1 ml of 120 μ M HBcAg-Lys-2cys-Mut in 20 mM Hepes, 150
15 mM NaCl pH 7.2 is reacted for 30 minutes with a 10 fold molar excess of SMPH (Pierce), diluted from a stock solution in DMSO, at 25 °C on a rocking shaker. The reaction solution is subsequently dialyzed twice for 2 hours against 1 L of 20 mM Hepes, 150 mM NaCl, pH 7.2 at 4 °C. The dialyzed HBcAg-Lys-2cys-Mut reaction mixture is then reacted with a five-fold molar excess of A β 1-6 peptide (sequence:
20 NH₂-DAEFRHGGC-CONH₂) (SEQ ID NO: 77) for 2 hours at 16 °C on a rocking shaker. Coupling products are analysed by SDS-PAGE.

C. Coupling of A β 1-6 peptide to Pili

A solution of 125 μ M Type-1 pili of *E.coli* in 20 mM Hepes, pH 7.4, is reacted for 60 minutes with a 50-fold molar excess of cross-linker SMPH (Pierce), diluted
25 from a stock solution in DMSO, at RT on a rocking shaker. The reaction mixture is desalted on a PD-10 column (Amersham-Pharmacia Biotech). The protein-containing

fractions eluting from the column are pooled, and the desalted derivatized pili protein is reacted with a five-fold molar excess of A β 1-6 peptide (sequence: NH₂-DAEFRHGGC-CONH₂) (SEQ ID NO: 77) for 2 hours at 16 °C on a rocking shaker. Coupling products are analysed by SDS-PAGE.

- 5 D. Immunization of mice with A β 1-6 peptide coupled to fr-capsid protein, HBcAg-Lys-2cys-Mut or pili

A β 1-6 peptide coupled to fr-capsid protein, HBcAg-Lys-2cys-Mut or pili as described above is injected s.c. in mice (3 mice) at day 0 and 14. Each mice (C57BL/6) is immunized with 10 μ g of vaccine diluted in PBS to 200 μ l. Mice are
10 retroorbitally bled on day 21, and the titer of the antibodies specific for the A β 1-6 peptide or A β 1-40 or A β 1-42 are measured by ELISA as described in Example 13.

EXAMPLE 19

Immunisation of rhesus monkeys with Q β hA β 1-6

In order to test induction of antibodies against human A β using a human A β 1-6
15 peptide based vaccine in the case where A β 1-6 is a self antigen, rhesus monkeys were immunized with Q β hA β 1-6, as the A β sequence is identical between humans and Rhesus monkeys. Q β hA β 1-6 vaccine was made as described in Example 13. Four Rhesus monkeys, between 10 and 15 years of age, were immunized at day 0 with 50 μ g of vaccine, and boosted twice at day 28 and 56 with 25 μ g of vaccine.
20 The monkeys were immunized subcutaneously in the back. The animals were bled at day 0 (prebleed), 42 and 70. 4 ml of blood were collected from the V. cephalica antebrachii. The titer of antibodies specific for A β 1-40 were measured by ELISA essentially as described in Example 13, using a secondary antibody specific for Monkey IgG.

25 As humans and rhesus monkeys share the same A β sequence, the generation of high titer antibodies in rhesus monkeys specific for A β 1-40 shows that immunization with hA β 1-6 coupled to Q β breaks tolerance against the self-antigen A β .

Furthermore, antibodies recognizing full length A β are generated with the coupled A β 1-6 fragment in primates.

The results of the ELISA are shown in Fig. 6. Plotted in the diagram are the titers of A β 1-40 specific antibodies measured in the sera of the 4 monkeys (1-4) immunized with Q β hA β 1-6 and the average of the titers of the 4 monkeys. The titers are represented as OD50 titers. OD50 is the dilution of the antibodies at which the signal reaches half of its maximal value. The maximal value (OD max) was obtained from a reference serum originating from a monkey immunised with Q β hA β 1-27 and recognizing A β 1-40 as well, and measured on the same ELISA plate.

Two monkeys (described above) were bled at day 97, 110, 117, 124, 138, 143, 152, 159, 166, and received a third boost with 25 μ g of vaccine at day 110. Sera were pooled (99 ml) and used for affinity purification of A β 1-6-specific antibodies. These antibodies were used for immunohistochemical staining at a concentration of 1.5 μ g/ml and a biotinylated secondary anti-monkey antibody was used for detection. Paraffine brain sections of 18 months old heterozygous APP23 mouse and an AD patient – Braak Stage III – were used for staining. Plaque-specific staining was observed both in APP23 mouse brain sections and in the AD patient brain sections (Fig. 7).

The result of the histological analysis is shown in Fig. 7 A and B. Depicted in Fig. 7 A is the staining of human APP transgenic mouse plaques (APP23 strain) with the above described affinity purified antiserum specific for A β 1-6. Fig. 7 B shows the staining of human AD plaques with the same purified antiserum. The purified antiserum was used at a concentration of 1.5 μ g/ml in both cases. Typical plaques are indicated by an arrow on both figures.

EXAMPLE 20

Coupling of murine A β 1-6 to AP205 VLP, immunisation of mice and analysis of immune responseA. Coupling of murine A β 1-6 peptide to AP205 VLP

5 The peptide murine A β 1-6 (mA β 1-6, sequence: NH₂-DAEFGHGHC-CONH₂ (SEQ ID NO: 78) was chemically synthesized; the initial NH₂ group indicates that the peptide has a free N-terminus, and the terminal NH₂ group indicates that the peptide has an amidated carboxy-terminus). AP205 VLP (expressed and purified as described in Example 1), in 20 mM Hepes, 150 mM NaCl, pH 8.0 (HBS, pH 8.0)
10 was reacted at a concentration of 2 mg/ml (determined in a Bradford assay), with 2.86 mM SMPH (Pierce, Rockford IL), diluted from a 100 mM stock in DMSO, for 30 minutes at room temperature (RT). The reaction mixture was then dialyzed twice against a 1000-fold volume of HBS, pH 7.4. at 4°C for two hours; the resulting dialyzed and derivatized AP205 VLP was flash frozen in liquid nitrogen and stored
15 at -20°C overnight. Derivatized AP205 VLP was diluted with one volume of 20 mM HBS, pH 7.4, and reacted 2 hours at 15°C under shaking with 719 μ M mA β 1-6 peptide diluted in the reaction mixture from a 50 mM stock in DMSO. The coupling reaction was dialyzed twice against a 1000-fold volume HBS, pH 7.4, for 2 hours and overnight. The dialyzed reaction mixture was flash frozen in liquid nitrogen in
20 aliquots for storage at -80°C until further use.

 An aliquot was thawed, and coupling of the mA β 1-6 peptide to the AP205 VLP subunits assessed by SDS-PAGE and the protein concentration measured in a Bradford assay. The result of the coupling reaction is shown in FIG. 8.

 FIG. 8 shows the SDS-PAGE analysis of the coupling reaction of mA β 1-6
25 peptide to AP205 VLP. The samples were run under reducing conditions on a 16% Tris-glycine gel and stained with coomassie brilliant blue. Lane 1 is the protein marker, with corresponding molecular weights indicated on the left border of the gel; lane 2, AP205 VLP protein; lane 3, derivatized AP205 VLP; lane 4, the supernatant of the coupling reaction of AP205 VLP to mA β 1-6 peptide; lane 5, the pellet of the
30 coupling reaction of AP205 VLP to mA β 1-6 peptide. No AP205 VLP subunits left

uncoupled could be detected on the gel, while bands corresponding to several peptides per subunits were visible, demonstrating a very high coupling efficiency. In particular, there is much more than one A β 1-6 peptide per AP205 VLP subunit.

5 B. Immunisation of mice with mA β 1-6 peptide coupled to AP205 VLP and analysis of immune response

AP205 VLP coupled to mA β 1-6 peptide was injected s.c. in mice (3 mice) at day 0 and 14. mA β 1-6 peptide was coupled to AP205 VLP as described above. Each mice (C57BL/6) was immunized with 25 μ g of vaccine diluted in PBS to 200 μ l. Mice were retroorbitally bled on day 21, and the titer of the antibodies specific for
10 the mA β 1-6 peptide were measured in an ELISA against mA β 1-6. The mA β 1-6 peptide was coupled to bovine RNase A using the chemical cross-linker sulfo-SPDP. ELISA plates were coated with preparations of RNase-mA β 1-6 at a concentration of 10 μ g/ml. The plates were blocked and then incubated with serially diluted mouse sera. Bound antibodies were detected with enzymatically labeled anti-
15 mouse IgG antibodies. As a control, preimmune sera of the same mice were also tested. The results are shown in FIG. 9.

FIG. 9 shows an ELISA analysis of the IgG antibodies specific for mA β 1-6 peptide in sera of mice immunized with the mA β 1-6 peptide coupled to AP205 VLP. The results are shown for the sera of the three immunized mice collected at day 21
20 (A1 d21 -A3 d21), the pre-immune serum is indicated as "pre imm" in the figure; the result for one pre-immune serum is shown. Comparison of the pre-immune serum with the sera of the mice immunized with mA β 1-6 coupled to AP205 VLP shows that a strong specific antibody response against peptide mA β 1-6, which is a self-antigen, could be obtained in the absence of adjuvant. Furthermore, coupling of a
25 self-peptide to AP205 VLP leads to break of tolerance against this peptide, and to a very high specific immune response. Thus, AP205 VLP is suitable for generating high antibody titers against A β peptides in the absence of adjuvant.

Immunisation with Q β hA β 1-6 reduces amyloid plaques in transgenic mice over-expressing the "Swedish/London" mutant amyloid precursor protein

This example demonstrates that immunization with Q β hA β 1-6 in a mouse
5 model developing Alzheimer's disease-like diffuse (Congo-Red negative) amyloid plaques, resulted in a massive reduction of plaque density in neocortical and subcortical brain areas. Histological occurrence of diffuse amyloid plaques is a prominent feature of AD brain pathology (Selkoe, 1994, Annu. Rev. Neurosci. 17:489-517) and, therefore, the example demonstrates that immunization with
10 Q β hA β 1-6 provides an effective approach for the treatment of Alzheimer's disease.

To evaluate the therapeutic efficacy of immunization with Q β -A β 1-6 transgenic mice over-expressing the "Swedish/London" mutant amyloid precursor protein under the control of the mouse Thy-1 promoter (APP24; K670N/M671L; V717I, patent No. WO980-36-4423) were used. This mouse strain is characterized
15 by a large number amyloid plaques in the neocortex, hippocampus, caudate putamen, and thalamus at an age of 18 months. Plaques can be first observed at an age of 9 months. Histologically, the amyloid plaques in APP24 mice are predominantly of a diffuse type, i.e. they are negative in Congo-Red staining. To a lesser degree, also compact amyloid plaques (Congo-Red positive) can be found.

Human A β 1-6 peptide coupled to Q β VLP (Q β hA β 1-6) was made as described
20 in Example 13. In terms of the experimental procedure followed, which is not necessary for describing or enabling the invention, APP24 transgenic mice 9.5 months of age were injected subcutaneously at day 0 with 25 μ g of Q β hA β 1-6 in phosphate-buffered saline (PBS) (administered as 2x100 μ l per mouse) (n=16) or as
25 negative controls with PBS (administered as 2x100 μ l per mouse) (n=9) or with Q β virus-like particle devoid of coupled antigen (n=11). Mice were subsequently injected 25 μ g of Q β hA β 1-6-vaccine, Q β , or PBS on day 15, 49, 76, 106, 140, 169, 200, 230, 259, and 291. Animals were bled 1-2 days before the first immunization (day 0) and on day 56, 90, 118, 188, 214, 246, and 272 via the tail vein. Blood serum
30 was also collected on day 305, at which time also brains were collected for histopathology (age of the mice at this time point: 19.5 months).

The titer of antibodies specific for A β 1-40 were measured by ELISA essentially as described in Example 13. The results of the ELISA are shown in Fig. 10. Plotted in the diagram are the titers of A β 1-40 or A β 1-42 specific antibodies measured in the sera of mice immunized with Q β hA β 1-6. The titers are represented as OD50% titers. OD50% is the dilution of the antibodies at which the signal reaches half of its maximal value. The maximal value (OD max) was obtained from a reference antibody recognizing A β 1-40 and Ab42, and measured on the same ELISA plate. All Q β hA β 1-6 immunized mice developed OD50% titers above 1:8000 (pre-immune serum titers were below 1:100) demonstrating a consistent antibody response to Q β hA β 1-6 even in old APP24 mice (Fig. 10). Median OD50% titers in the immunized group were in the range of 1:20'000 to 1:50'000 throughout the immunization period.

For quantification of amyloid plaques, brains were fixed by immersion in 4% formaldehyde in 0.1 M PBS at 4°C. After dehydration with ethanol, brains were embedded in paraffin and cut sagittally with a microtome at 4 μ m thickness. Sections were mounted onto super frost slides and dried at 37°C. Sections were washed in PBS and antigenicity enhanced by microwave heating at 90°C for 3 minutes in 0.1 M citric acid buffer. NT11 antisera (anti A β 1-40, Sturchler-Pierrat et al., 1997, Proc. Natl. Acad. Sci. 94: 13287-13292) were diluted 1:1000 in PBS with 3% goat serum and incubated over night at 4°C. Following rinsing, sections were incubated for 1 hour with biotinylated anti rabbit IgG secondary antibody (BA1000, Vector Laboratories) diluted 1:200 in PBS. After rinsing, sections were further processed with the avidin-biotin-peroxidase technique (ABC-Elite Kit PK6100; Vector Laboratories). Finally, sections were reacted with Diaminobenzidine (DAB) metal enhanced substrate (Boehringer, Code 1718096), counterstained with Hemalum, dehydrated, cleared in Xylene and cover slipped. Systematic-random series of brain sections at three different anatomical planes per animal were used for the analysis. Amyloid plaques were quantified using an MCID image analyzer (Imaging Research, Brock University, Ontario-Canada, Program Version M5 elite). The microscopic image was digitized by use of a Xillix black and white CCD TV camera and stored with 640x480 pixel resolution at 256 gray levels. The pixel size was calibrated using an object micrometer at 5x magnification (Leica Neoplan Objective). Using a motor

driven microscope stage for exact positioning of adjacent object fields the entire neocortex and olfactory nucleus of each section was analysed. For each object field the anatomical area was defined by manual outline. For each individual section the sample area was defined by manual threshold setting (grey level) between
5 immunopositive amyloid plaques and tissue background. Isolated tissue artifacts were excluded by manual outline. Raw data are measured as individual counts (amyloid deposits) and proportional area values (immunopositive amyloid/cortex or olfactory nucleus).

Data of each mouse were normalized to number of deposits (plaques) per mm²
10 and total plaque area in % of the entire neocortex. Q β hA β 1-6 immunized mice revealed a dramatic reduction of amyloid deposits in the cortex and subcortical areas as compared to either PBS or Q β injected control groups (Fig. 11). Both the median number of deposits and the total plaque area were highly significantly reduced between 80-98 % compared to the PBS group in the cortex, caudate putamen,
15 hippocampus, and thalamus ($p < 0.001$ vs. PBS-group, Mann-Whitney test; Fig. 12).

In a second study, APP24 transgenic mice 13.5 months of age were injected subcutaneously at day 0 with 25 μ g of Q β hA β 1-6 in phosphate-buffered saline (PBS) (administered as 2x100 μ l per mouse) (n=15) or as negative controls with PBS (administered as 2x100 μ l per mouse) (n=15). Mice were subsequently injected 25
20 μ g of Q β hA β 1-6-vaccine, or PBS on day 16, 46, 76, 109, 140, and 170. Animals were bled 1-2 days before the first immunization (day 0) and on day 31, 59, 128, and 154 via the tail vein. Blood serum was also collected on day 184, at which time also brains were collected for histopathology (age of the mice at this time point: 19.5 months). The titer of antibodies specific for A β 1-40 were determined and expressed
25 as described above and again all immunized mice were found to respond to Q β hA β 1-6 immunization with serum OD50% titers at least above 1:2000 (not shown). Median OD50% titers were in the range of 1:10'000 to 1:50'000 throughout the immunization period. Quantification of amyloid deposits was done as described above. Compared to the experiment where immunization was initiated earlier (i.e. at
30 an age of 9.5 months) the reduction of plaque deposit number (-55 %) and area (- 32 %) was less dramatic in the neocortex, but still very pronounced (Fig. 13) and highly significant ($p > 0.001$ vs. PBS, Mann-Whitney test). In subcortical areas plaque

deposit number and area were reduced by 60-90 % in the to Q β hA β 1-6 immunized group. The more pronounced effect in these areas as compared to the cortex is probably related to the more protracted time course of plaque formation in these areas.

5 Taken together, both experiments demonstrate that Q β hA β 1-6 immunization in transgenic mice over-expressing the “Swedish/London” mutant amyloid precursor protein dramatically reduces the occurrence of amyloid deposits in these mice.

Figure 10: Serum anti A β 40/42 antibody titers (OD50%) in transgenic mice over-expressing the “Swedish/London” mutant amyloid precursor protein. Mice were
10 immunized with Q β hA β 1-6 between 9.5 and 19 months of age. Shown are individual values (black dots) and box plots, where the ends of the boxes define the 25th and 75th percentiles, with a line at the median and error bars defining the 10th and 90th percentiles (outliers are shown as dots).

Figure 11: Immunohistochemical staining of amyloid plaques in sagittal brain
15 sections. The sagittal brain section of a transgenic mouse over-expressing the “Swedish/London” mutant amyloid precursor protein immunized with Q β (A) or Q β hA β 1-6 (B) vaccine is shown in the Figure.

Figure 12: Quantification of plaque deposition in transgenic mice over-expressing the “Swedish/London” mutant amyloid precursor protein after
20 immunization between 9.5 and 19 months of age. (A) Cortical plaque density. (B) Cortical plaque area. (C) Plaque density in the caudate putamen. (D) Plaque area in the caudate putamen. (E) Plaque density in the hippocampus. (F) Plaque area in the hippocampus. (G) Plaque density in the thalamus. (H) Plaque area in the thalamus. Plaque density is expressed in plaques/mm², plaque area in percent of tissue area covered by amyloid beta. Data are shown as individual values (black dots) and box
25 plot. The ends of the boxes define the 25th and 75th percentiles, with a line at the median and error bars defining the 10th and 90th percentiles. ** p<0.001 (Mann Whitney Rank Sum Test). PBS, n=9, Q β , n=11, Q β hA β 1-6, n=16.

Immunisation with Q β hA β 1-6 reduces amyloid plaques in transgenic mice over-expressing the "Swedish" mutant amyloid precursor protein.

This example demonstrates that immunization with Q β hA β 1-6 provides an effective approach for the treatment of Alzheimer's disease even when the immunization is initiated in a very advanced stage of amyloid plaque pathology. The amyloid plaque deposition process in the AD mouse model used in this example starts already at an age about 6 months (Sturchler-Pierrat et al., 1997, Proc. Natl. Acad. Sci. 94: 13287-13292). In the study described herein, immunization with Q β hA β 1-6 was initiated at an age of 18 months, where already a high number of compact plaques had been formed in the cortex. The example also demonstrates the ability of Q β hA β 1-6 to induce A β 40/42 antibodies in very aged animals (no non-responders in 19 immunized mice).

To evaluate the therapeutic effects of immunization with Q β hA β 1-6 transgenic mice over-expressing the "Swedish" mutant amyloid precursor protein (APP23; K670N/M671L, Sturchler-Pierrat et al., 1997, Proc. Natl. Acad. Sci. 94: 13287-13292) were used. The Alzheimer's-like pathology in these mice has been extensively characterized (Calhoun et al., 1998, Nature 395: 755-756; Phinney et al., 1999, J. Neurosci. 19: 8552-8559; Bondolfi et al., 2002, J. Neurosci. 22: 515-522).

Human A β 1-6 peptide coupled to Q β VLP (Q β hA β 1-6) was made as described in Example 13. In terms of the experimental procedure followed, which is not necessary for describing or enabling the invention, APP23 transgenic mice 18 months of age were injected subcutaneously at day 0 with 25 μ g of Q β hA β 1-6 dissolved in phosphate-buffered saline (administered as 2x100 μ l per mouse) (n=19) or phosphate-buffered saline as a negative control (n=17) and boosted on day 13, 27-34, 61-63, 90-96, and 123-130 with 25 μ g of vaccine. Animals were bled 1-2 days before the first immunization (day 0) and on day 41-45, and day 68 via the tail vein. Blood serum was also collected on day 152-154, at which time also brains were collected for histopathology (age of the mice at this time point: 23 months).

The titer of antibodies specific for A β 1-40 were measured by ELISA essentially as described in Example 13 and the results expressed as described in Example 21. The results of the ELISA are shown in Fig. 14. All Q β hA β 1-6

immunized mice developed OD50% titers above 1:2000 (pre-immune serum titers were below 1:100) demonstrating a consistent antibody response to Q β -A β 1-6 even in very old mice (Fig. 14). Median OD50% titers were in the range of 1:9'000 to 1:20'000 throughout the immunization period.

5 Quantification of amyloid plaques was done as described in Example 21. Data of each mouse were normalized to number of deposits (plaques) per mm² and total plaque area in % of the entire neocortex. Q β hA β 1-6 immunized mice revealed a smaller number of deposits in the cortex (Figure 15, Fig. 16), mostly due to a reduction of small sized plaques. Compared to the non-immunized group the median
10 plaque number was reduced by 33 % in the Q β hA β 1-6 immunized group ($p < 0.001$ vs PBS-group, Mann-Whitney test). Since mostly small-sized plaques were affected the reduction of the total plaque area was moderate and amounted to 10 % ($p < 0.01$ vs. PBS group, Mann-Whitney test).

Figure 14: Serum anti A β 40/42 antibody titers (OD50%) in transgenic mice
15 over-expressing the "Swedish" mutant amyloid precursor protein. Mice were immunized with Q β hA β 1-6 between 18 and 23 months of age. Shown are individual values (black dots) and box plots, where the ends of the boxes define the 25th and 75th percentiles, with a line at the median and error bars defining the 10th and 90th percentiles (outliers are shown as dots).

20 Figure 15: Immunohistochemical staining of amyloid plaques in sagittal brain sections. Arrows point to small sized deposits. Shown in the Figure is a sagittal brain section from a transgenic mouse over-expressing the "Swedish" mutant amyloid precursor protein immunized with PBS (A) or Q β -A β 1-6 (B).

Figure 16: Quantification of plaque deposition in transgenic mice over-
25 expressing the "Swedish" mutant amyloid precursor protein after immunization between 18 and 23 months of age. (A) Cortical plaque density. (B) Cortical plaque area. Plaque density is expressed in plaques/mm², plaque area in percent of tissue area covered by amyloid beta. Data are shown as individual values (black dots) and box plot. The ends of the boxes define the 25th and 75th percentiles, with a line at the
30 median and error bars defining the 10th and 90th percentiles. ** $p < 0.001$ (Mann-Whitney Rank Sum Test). PBS, n=17, Q β hA β 1-6, n=19.

Having now fully described the present invention in some detail by way of illustration and example for purposes of clarity of understanding, it will be obvious to one of ordinary skill in the art that the same can be performed by modifying or changing the invention within a wide and equivalent range of conditions, formulations and other parameters without affecting the scope of the invention or any
5 specific embodiment thereof, and that such modifications or changes are intended to be encompassed within the scope of the appended claims.

All publications, patents and patent applications mentioned in this specification are indicative of the level of skill of those skilled in the art to which this invention
10 pertains, and are herein incorporated by reference to the same extent as if each individual publication, patent or patent application was specifically and individually indicated to be incorporated by reference.

CLAIMS:

1. A composition comprising:
- (a) a core particle with at least one first attachment site; and
 - (b) at least one antigen or antigenic determinant with at least one
5 second attachment site,
- wherein said antigen or antigenic determinant is a A β 1-6 peptide, and
wherein said second attachment site being selected from the group
consisting of:
- (i) an attachment site not naturally occurring with said
10 antigen or antigenic determinant; and
 - (ii) an attachment site naturally occurring with said antigen
or antigenic determinant,
- wherein said second attachment site is capable of association to said first
attachment site; and wherein said A β 1-6 peptide and said core particle
15 interact through said association to form an ordered and repetitive antigen
array.
2. The composition of claim 1, wherein said core particle is selected
from the group consisting of:
- 20 i) a virus;
 - ii) a virus-like particle;
 - iii) a bacteriophage;
 - iv) a virus-like particle of a RNA-phage;
 - v) a bacterial pilus;
 - 25 vi) a viral capsid particle; and
 - vii) a recombinant form of (i), (ii), (iii), (iv), (v) or (vi).
3. The composition of any one of claim 1 or 2, wherein said core particle
comprises, preferably is, a virus-like particle, wherein preferably said virus-like
30 particle is a recombinant virus-like particle.

4. The composition of any one of claim 2 or 3, wherein said virus-like particle comprises recombinant proteins, or fragments thereof, selected from the group consisting of:

- 5 (a) recombinant proteins of Hepatitis B virus;
- (b) recombinant proteins of measles virus;
- (c) recombinant proteins of Sindbis virus;
- (d) recombinant proteins of Rotavirus;
- (e) recombinant proteins of Foot-and-Mouth-Disease virus;
- 10 (f) recombinant proteins of Retrovirus;
- (g) recombinant proteins of Norwalk virus;
- (h) recombinant proteins of Alphavirus;
- (i) recombinant proteins of human Papilloma virus;
- (j) recombinant proteins of Polyoma virus;
- 15 (k) recombinant proteins of bacteriophages;
- (l) recombinant proteins of RNA-phages;
- (m) recombinant proteins of Ty;
- (n) recombinant proteins of Q β -phage;
- (o) recombinant proteins of GA-phage;
- 20 (p) recombinant proteins of fr-phage;
- (q) recombinant proteins of AP205 phage; and
- (q) fragments of any of the recombinant proteins from (a) to (q).

25 5. The composition of any one of claims 2 to 3, wherein said virus-like particle is Hepatitis B virus core antigen.

6. The composition of any one of claims 2 to 3, wherein said virus-like particle comprises, or alternatively consists of, recombinant proteins, or fragments
30 thereof, of a RNA-phage.

7. The composition of claim 6, wherein said RNA-phage is selected from the group consisting of:

- (a) bacteriophage Q β ;
- (b) bacteriophage R17;
- 5 (c) bacteriophage fr;
- (d) bacteriophage GA;
- (e) bacteriophage SP;
- (f) bacteriophage MS2;
- (g) bacteriophage M11;
- 10 (h) bacteriophage MX1;
- (i) bacteriophage NL95;
- (k) bacteriophage f2;
- (l) bacteriophage PP7; and
- (m) bacteriophage AP205.

15

8. The composition of any one of claims 2 to 3, wherein said virus-like particle comprises, or alternatively consists of, recombinant proteins, or fragments thereof, of RNA-phage Q β .

20 9. The composition of any one of claims 2 to 3, wherein said virus-like particle comprises, or alternatively consists of, recombinant proteins, or fragments thereof, of RNA-phage fr.

25 10. The composition of any one of claims 2 to 3, wherein said virus-like particle comprises, or alternatively consists of, recombinant proteins, or fragments thereof, of RNA-phage AP205.

30 11. The composition of any one of claims 3 to 10, wherein the recombinant proteins comprise, or alternatively consist essentially of, or alternatively consist of coat proteins of RNA phages.

12. The composition of claim 11, wherein said coat proteins of RNA phages having an amino acid are selected from the group consisting of:

- (a) SEQ ID NO:4;
- (b) a mixture of SEQ ID NO:4 and SEQ ID NO:5;
- 5 (c) SEQ ID NO:6;
- (d) SEQ ID NO:7;
- (e) SEQ ID NO:8;
- (f) SEQ ID NO:9;
- (g) a mixture of SEQ ID NO:9 and SEQ ID NO:10;
- 10 (h) SEQ ID NO:11;
- (i) SEQ ID NO:12;
- (k) SEQ ID NO:13;
- (l) SEQ ID NO:14;
- (m) SEQ ID NO:15;
- 15 (n) SEQ ID NO:16; and
- (o) SEQ ID NO:28.

13. The composition of any one of claims 3 to 10, wherein the recombinant proteins comprise, or alternatively consist essentially of, or alternatively
20 consist of mutant coat proteins of RNA phages.

14. The composition of claim 13, wherein said RNA-phage is selected from the group consisting of:

- (a) bacteriophage Q β ;
- 25 (b) bacteriophage R17;
- (c) bacteriophage fr;
- (d) bacteriophage GA;
- (e) bacteriophage SP;
- (f) bacteriophage MS2;
- 30 (g) bacteriophage M11;
- (h) bacteriophage MX1;
- (i) bacteriophage NL95;

- (k) bacteriophage f2;
- (l) bacteriophage PP7; and
- (m) bacteriophage AP205.

5 15. The composition of any one of claims 13 to 14, wherein said mutant coat proteins of said RNA phage have been modified by removal of at least one lysine residue by way of substitution.

10 16. The composition of any one of claims 13 to 14, wherein said mutant coat proteins of said RNA phage have been modified by addition of at least one lysine residue by way of substitution.

15 17. The composition of any one of claims 13 to 14, wherein said mutant coat proteins of said RNA phage have been modified by deletion of at least one lysine residue.

20 18. The composition of any one of claims 13 to 14, wherein said mutant coat proteins of said RNA phage have been modified by addition of at least one lysine residue by way of insertion.

 19. The composition of any of the preceding claims, wherein said second attachment site is capable of association to said first attachment site through at least one covalent bond.

25 20. The composition of any of the preceding claims, wherein said second attachment site is capable of association to said first attachment site through at least one non-peptide bond.

30 21. The composition of any of the preceding claims, wherein said A β 1-6 peptide is fused to said core particle.

22. The composition of any of the preceding claims, wherein said A β 1-6 peptide is selected from the group consisting of:

- (a) human A β 1-6 peptide having an amino acid sequence of SEQ ID NO:75;
- 5 (b) murine A β 1-6 peptide having an amino acid sequence of SEQ ID NO:76;
- (c) primate A β 1-6 peptide having an amino acid sequence of SEQ ID NO:84;;
- (d) rabbit A β 1-6 peptide having an amino acid sequence of SEQ ID NO:85;
- 10 (e) xenopus laevis A β 1-6 peptide having an amino acid sequence of SEQ ID NO:86;
- (f) rat A β 1-6 peptide having an amino acid sequence of SEQ ID NO:87; and
- 15 (g) guinea pig A β 1-6 peptide having an amino acid sequence of SEQ ID NO:88.

23. The composition of any of the preceding claims, wherein said A β 1-6 peptide has an amino acid sequence of SEQ ID NO:75.

20

24. The composition of any of the preceding claims further comprising an amino acid linker, wherein said amino acid linker comprises, or alternatively consists of, said second attachment site.

25. The composition of any of the preceding claims, wherein said second attachment or said amino acid linker with said second attachment site is bound to said A β 1-6 peptide at its C-terminus.

26. The composition of any of the preceding claims, wherein said second attachment site or said amino acid linker with said second attachment site is selected from the group consisting of:

- (a) GGC;

30

- (b) GGC-CONH₂;
(c) GC;
(d) GC-CONH₂;
(e) C; and
5 (f) C-CONH₂.

27. The composition of any of the preceding claims, wherein said A β 1-6 peptide with said at least second attachment site is NH₂-DAEFRHGGC-CONH₂ (SEQ ID NO: 77).

10

28. The composition of claim 27, wherein said virus-like particle is a virus-like particle of RNA-phage Q β coat protein.

29. A pharmaceutical composition comprising:
15 (a) the composition of claim 1; and
(b) an acceptable pharmaceutical carrier.

30. The pharmaceutical composition of claim 29 further comprising an adjuvant.

20

31. The pharmaceutical composition of claim 29 or claim 30, wherein said vaccine composition is devoid of an adjuvant.

32. A vaccine composition comprising the composition of any of the
25 preceding claims.

33. The vaccine composition of claim 32, further comprising an adjuvant.

34. The vaccine composition of claim 32 or claim 33, wherein said
30 vaccine composition is devoid of an adjuvant.

35. The vaccine composition of any one of claims 32 to 34, wherein said virus-like particle comprises recombinant proteins, or fragments thereof, of a RNA-phage.

5 36. The vaccine composition of any one of claims 32 to 35, wherein said virus-like particle comprises recombinant proteins or fragments thereof, of RNA-phage Q β .

37. The vaccine composition of any one of claims 32 to 35, wherein said
10 virus-like particle comprises recombinant proteins, or fragments thereof, of RNA-phage fr.

38. The vaccine composition of any one of claims 32 to 35, wherein said virus-like particle comprises recombinant proteins, or fragments thereof, of RNA-
15 phage AP205.

39. The vaccine composition of any one of claims 32 to 38, wherein said A β 1-6 peptide is selected from the group consisting of:

- 20 (a) human A β 1-6 peptide having an amino acid sequence of SEQ ID NO:75;
- (b) murine A β 1-6 peptide having an amino acid sequence of SEQ ID NO:76;
- (c) primate A β 1-6 peptide having an amino acid sequence of SEQ ID NO:84;;
- 25 (d) rabbit A β 1-6 peptide having an amino acid sequence of SEQ ID NO:85;
- (e) xenopus laevis A β 1-6 peptide having an amino acid sequence of SEQ ID NO:86;
- (f) rat A β 1-6 peptide having an amino acid sequence of SEQ ID
30 NO:87; and
- (g) guinea pig A β 1-6 peptide having an amino acid sequence of SEQ ID NO:88.

40. The vaccine composition of any one of claims 32 to 39 further comprising an amino acid linker, wherein said amino acid linker comprises, or alternatively consists of, said second attachment site.

5

41. The vaccine composition of claim 40, wherein said amino acid linker with said second attachment site is bound to said A β 1-6 peptide at its C-terminus.

42. The vaccine composition of any one of claims 40 to 41, wherein said amino acid linker with said second attachment site is selected from the group consisting of:

- (a) GGC;
- (b) GGC-CONH₂;
- (c) GC;
- 15 (d) GC-CONH₂;
- (e) C; and
- (f) C-CONH₂.

43. The vaccine composition of any one of claims 32 to 42, wherein said antigen or antigenic determinant with said at least second attachment site is NH₂-DAEFRHGGC-CONH₂ (SEQ ID NO: 77).

44. The vaccine composition of claim 43, wherein said virus-like particle is a virus-like particle of RNA-phage Q β coat protein.

25

45. A process for producing a composition of any of the preceding claims comprising:

- (a) providing a core particle with at least one first attachment site;
- (b) providing at least one antigen or antigenic determinant with at least one second attachment site,

30

wherein said antigen or antigenic determinant is a A β 1-6 peptide, and
wherein said second attachment site being selected from the group
consisting of:

- 5 (i) an attachment site not naturally occurring with said
antigen or antigenic determinant; and
(ii) an attachment site naturally occurring with said antigen
or antigenic determinant; and

wherein said second attachment site is capable of association to said
first attachment site; and

- 10 (c) combining said core particle and said at least one antigen or
antigenic determinant, wherein said antigen or antigenic
determinant and said core particle interact through said
association to form an ordered and repetitive antigen array.

- 15 46. A method of immunization comprising administering the composition
of any of the preceding claims to an animal or human.

47. The method of immunization of claim 46, wherein said antigen or
antigenic determinant is a self-antigen.

- 20 48. The method of immunization of any one of claims 46 to 47, wherein
said animal is a human.

49. The method of immunization of any one of claims 46 to 48, wherein
25 said antigen or antigenic determinant is human A β 1-6 peptide.

50. Composition of any one of claims 1 to 44 for use as a medicament.

51. Use of a composition of any one of claims 1 to 44 for the manufacture
30 of a medicament for treatment of Alzheimer and related diseases.

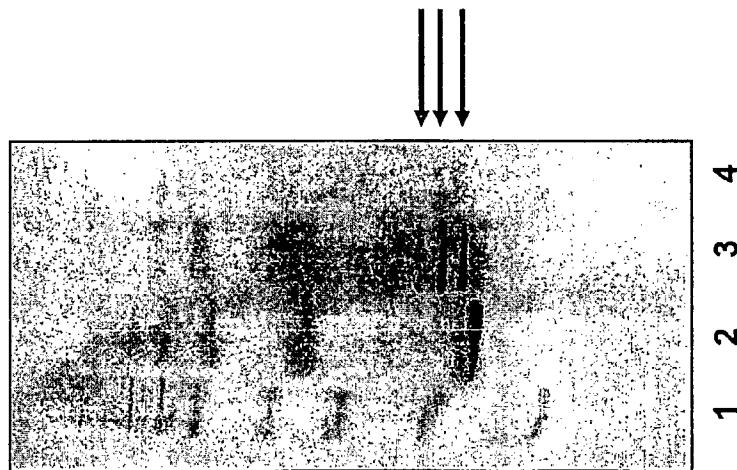


FIG. 1

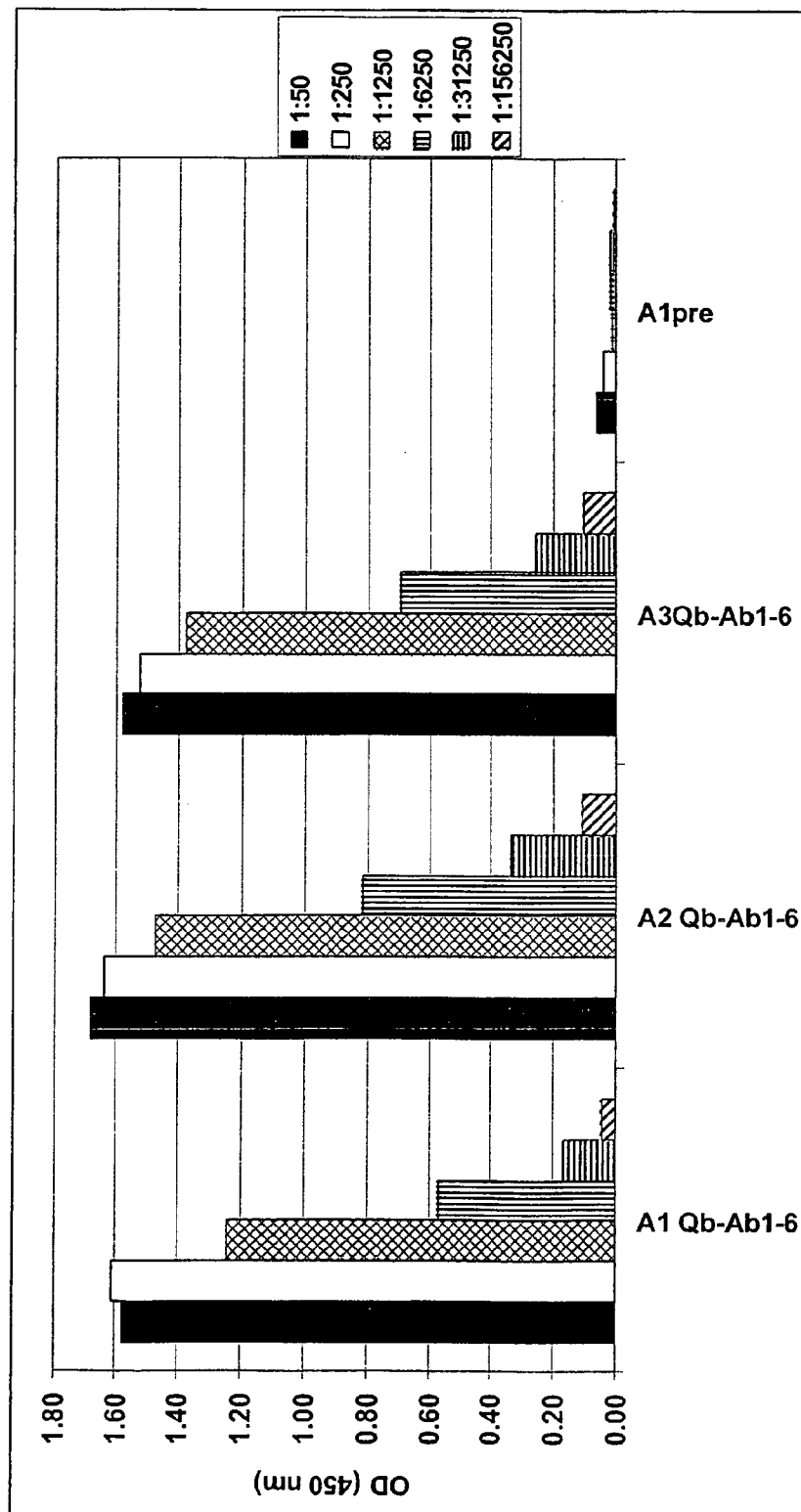


FIG. 2

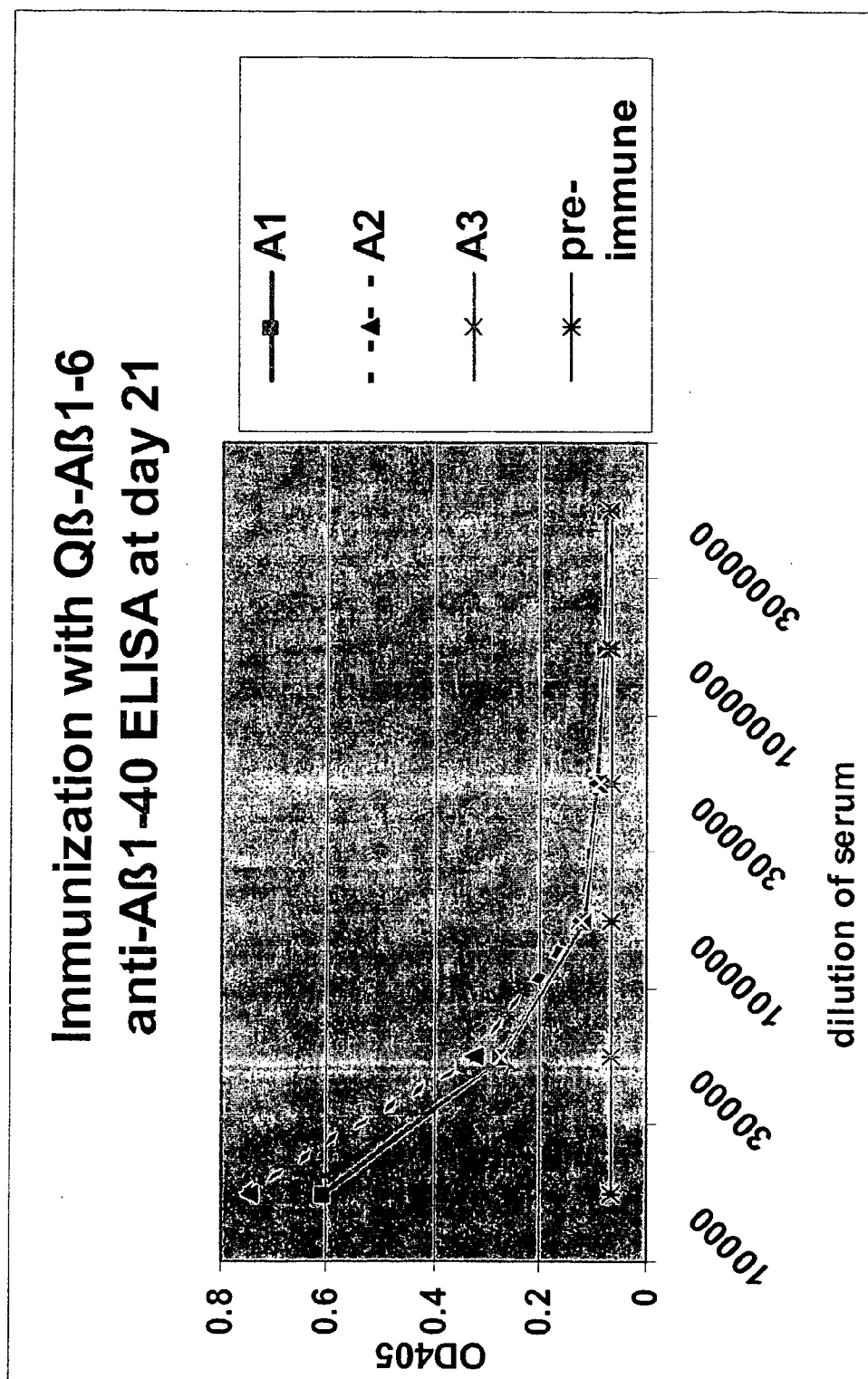


FIG. 3

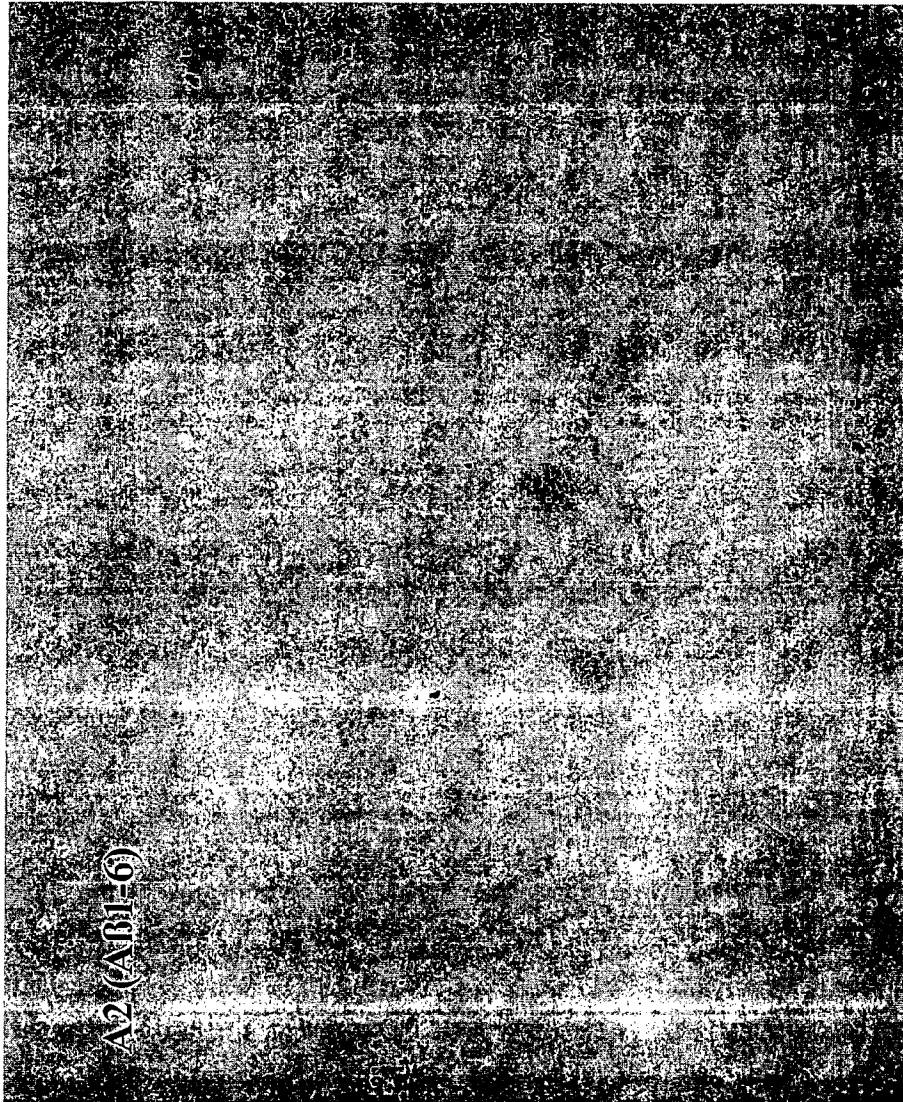


FIG. 4A

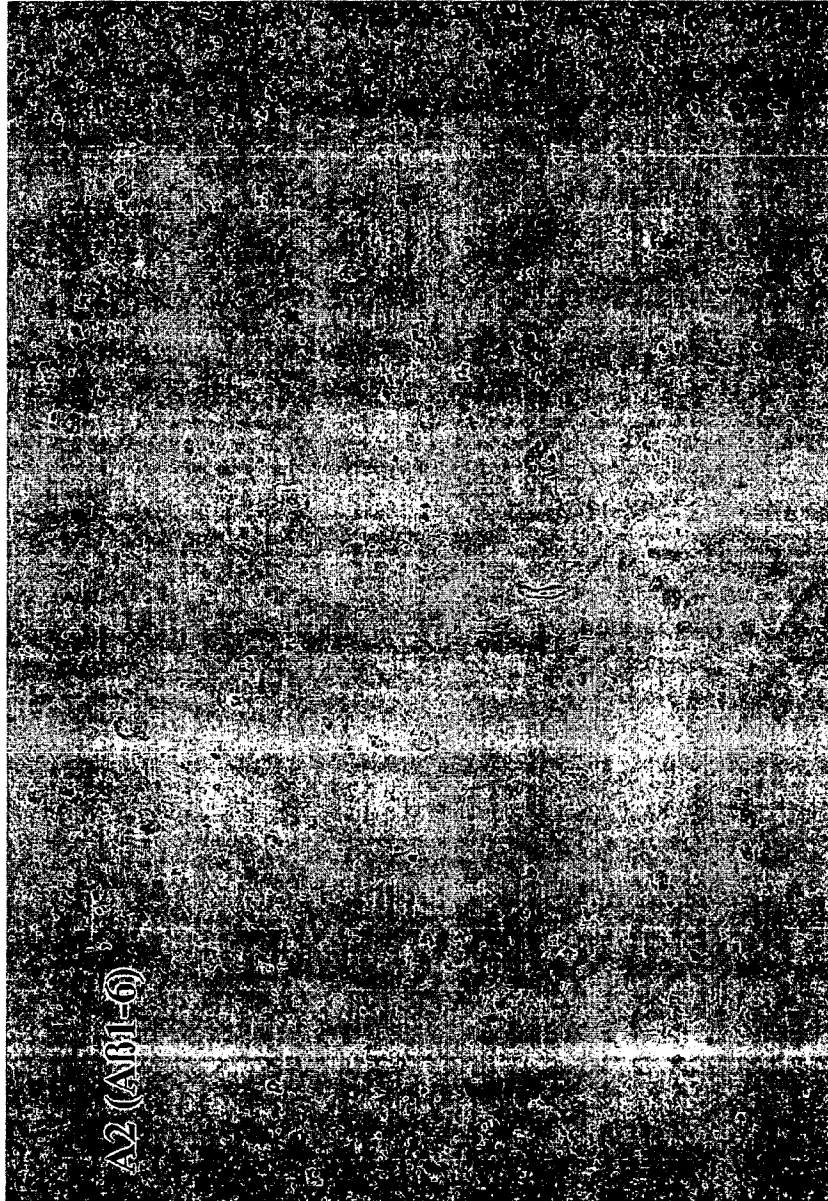


FIG. 4B

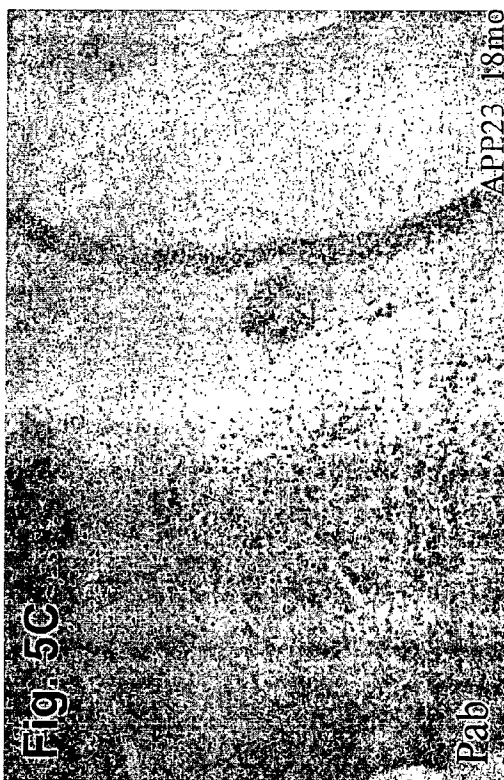
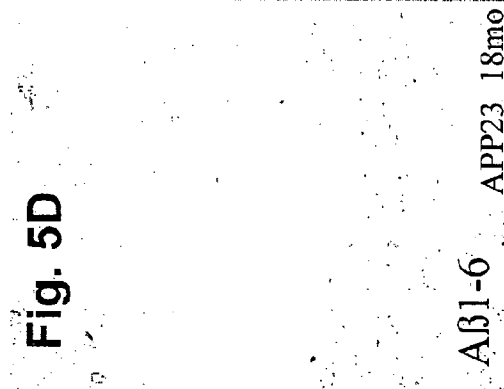
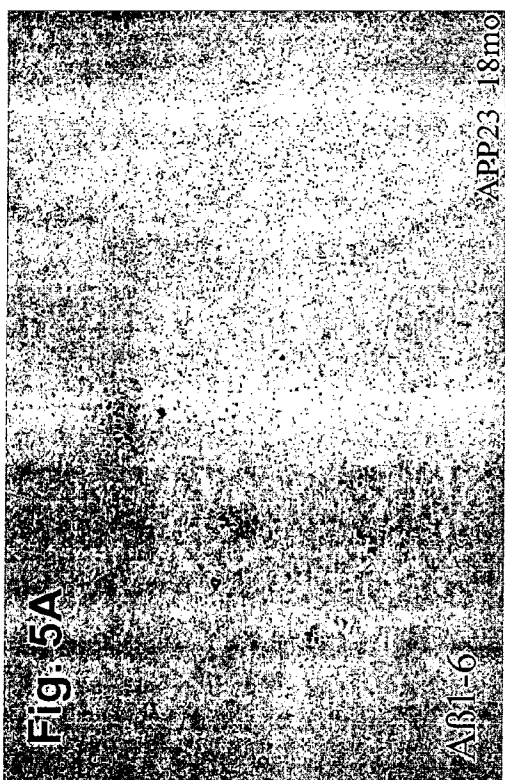
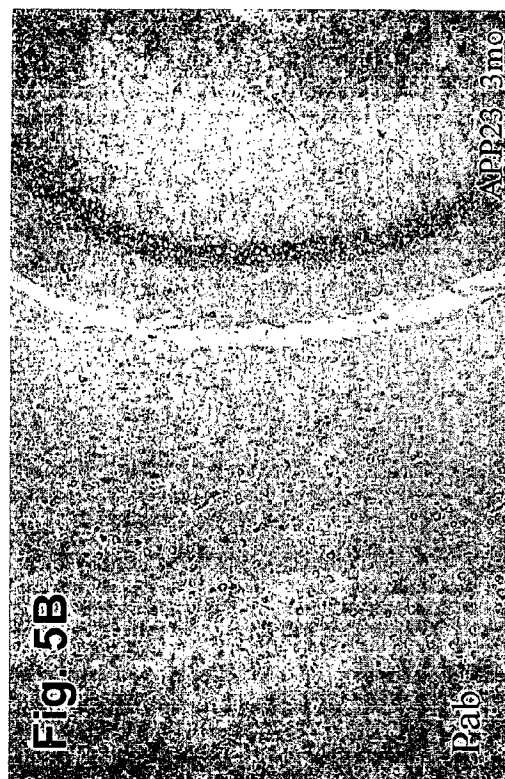
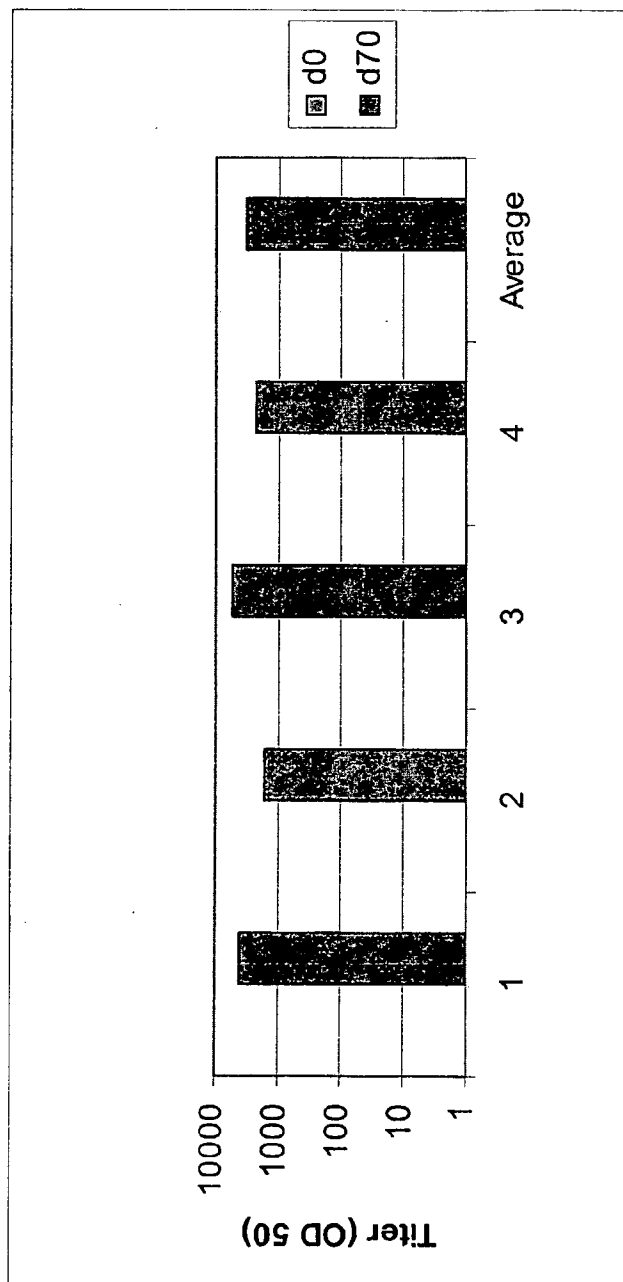


Fig. 5

**Fig. 6**

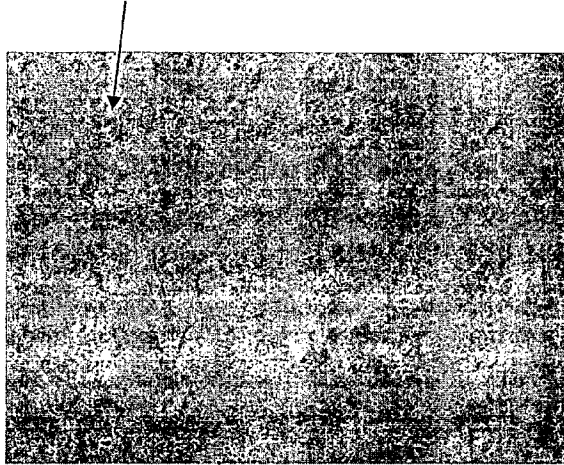


Fig. 7B

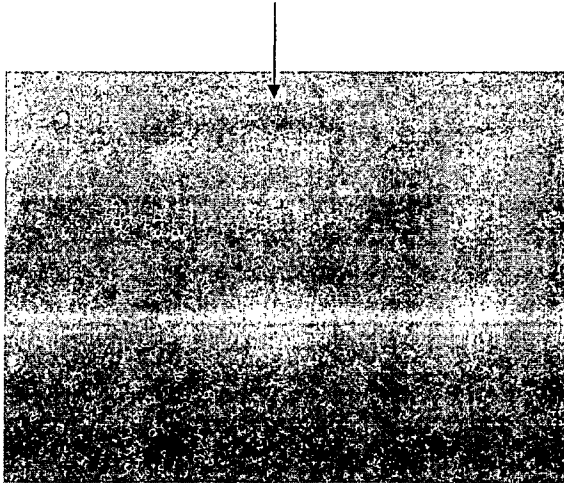


Fig. 7A

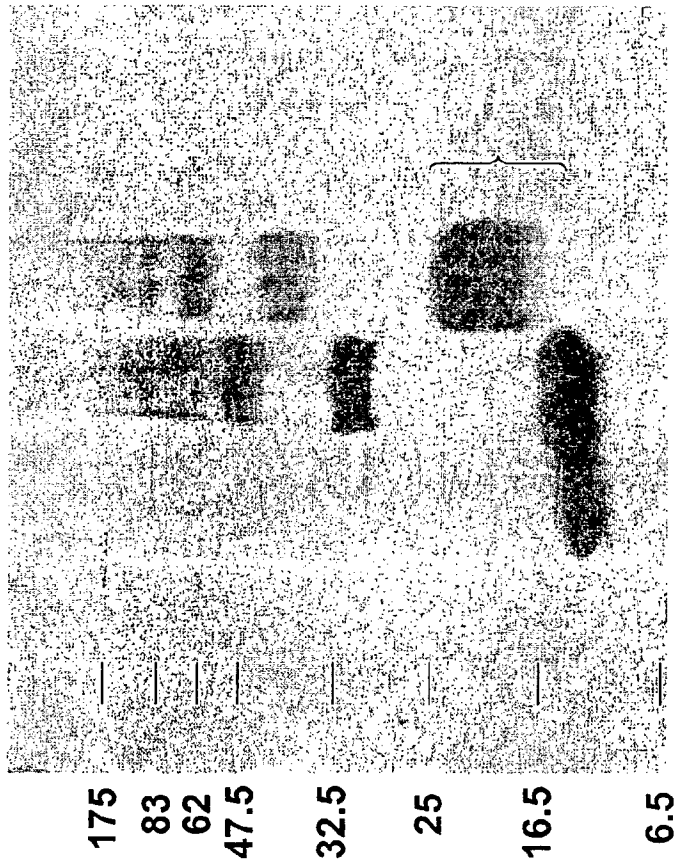


Fig. 8

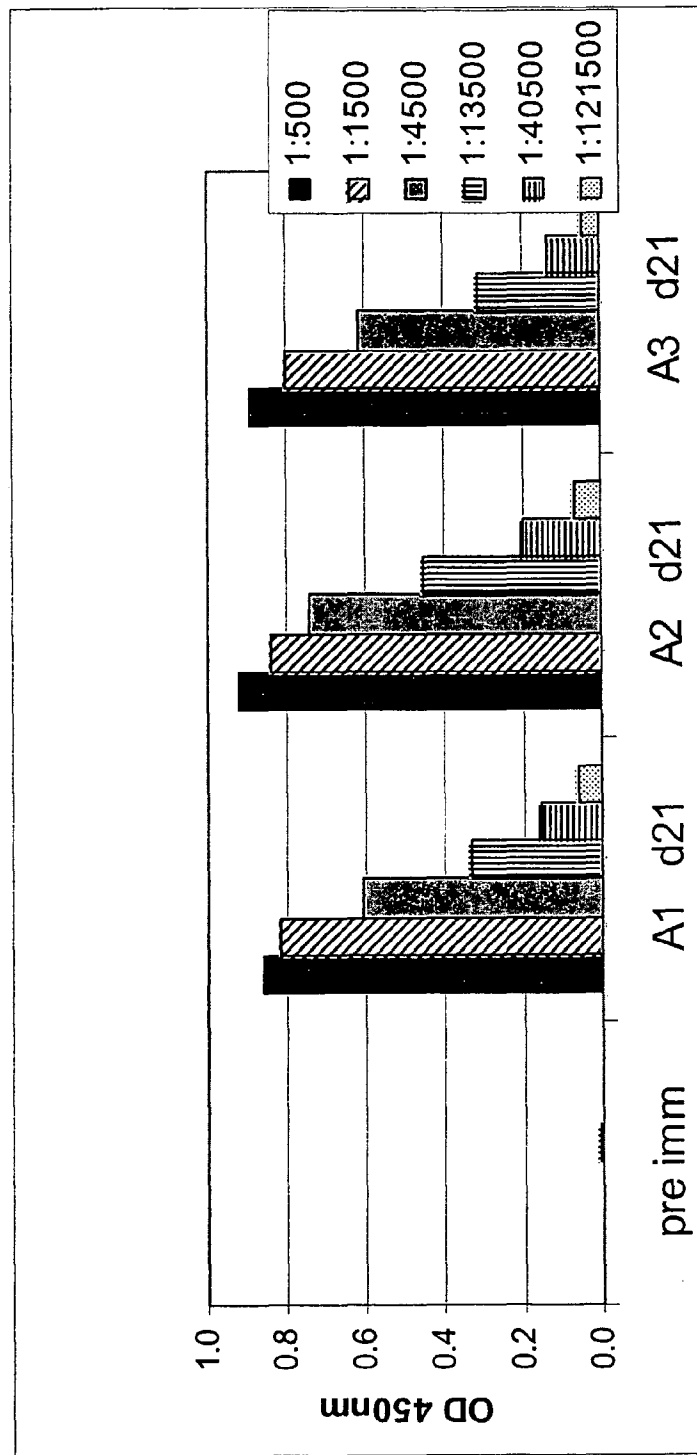


Fig. 9

11/23

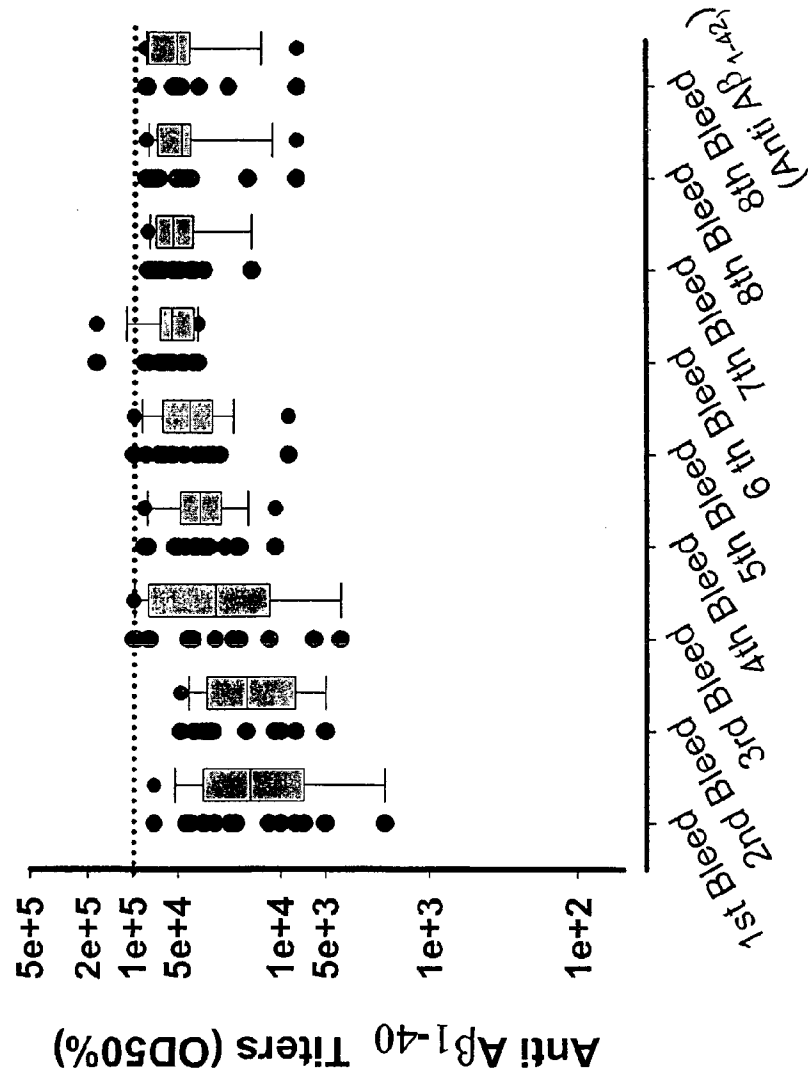


Fig. 10

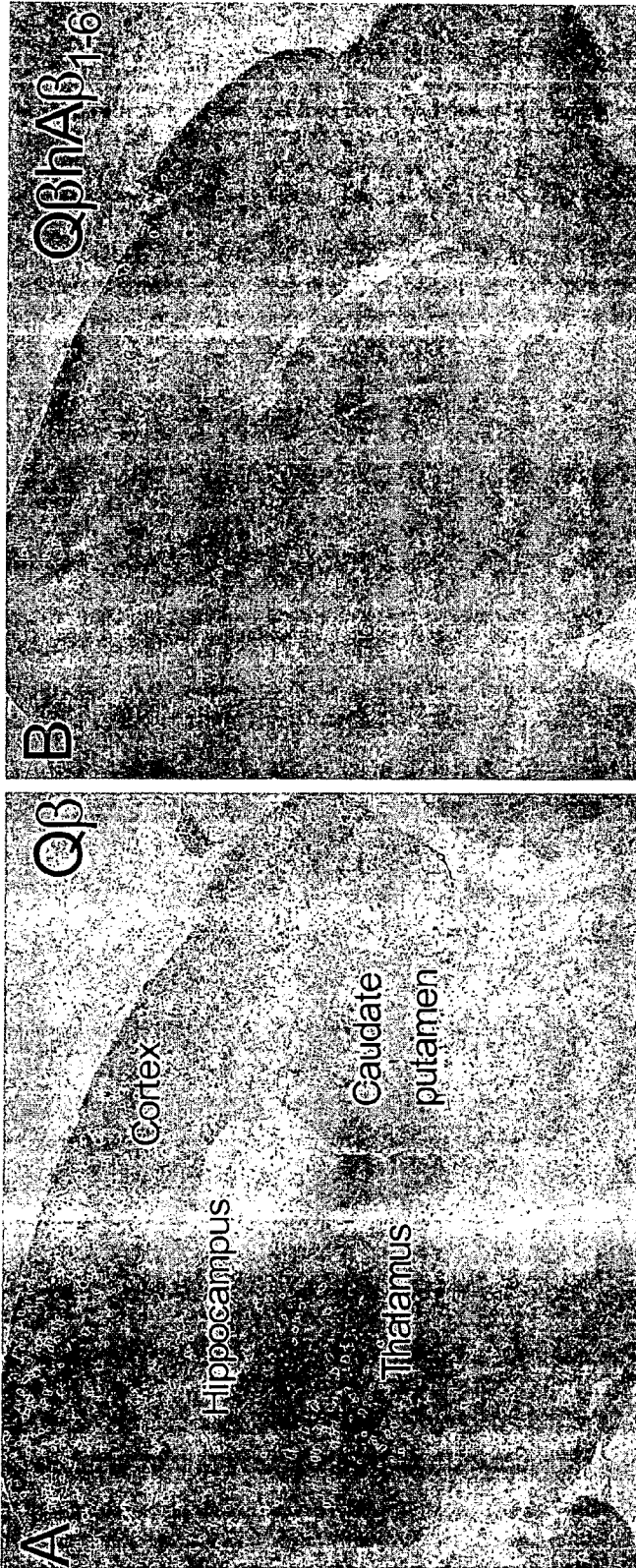


Fig. 11B

Fig. 11A

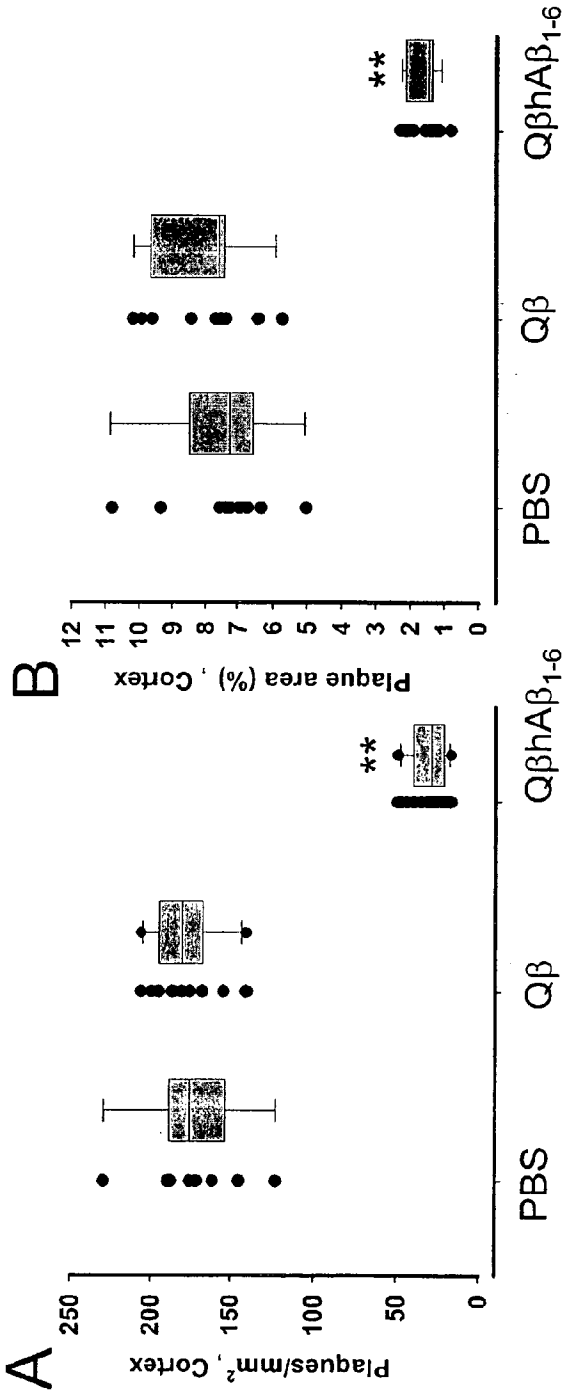


Fig. 12A

Fig. 12B

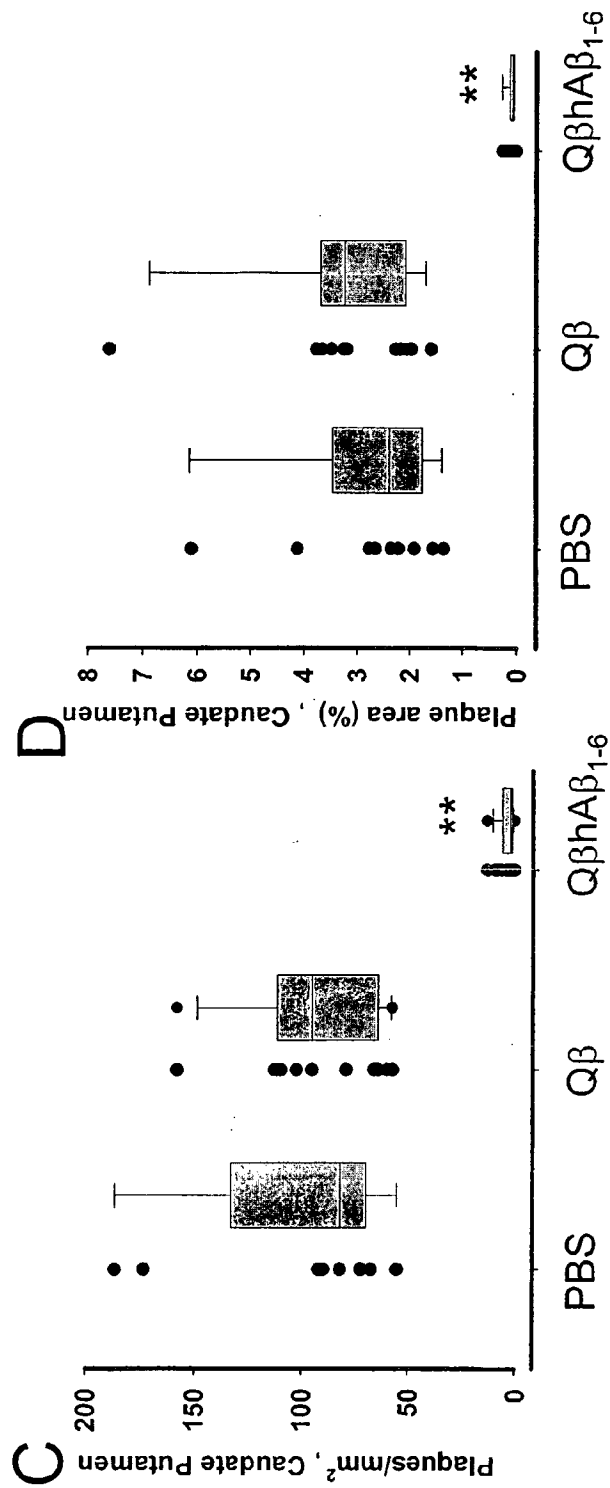


Fig. 12C

Fig. 12D

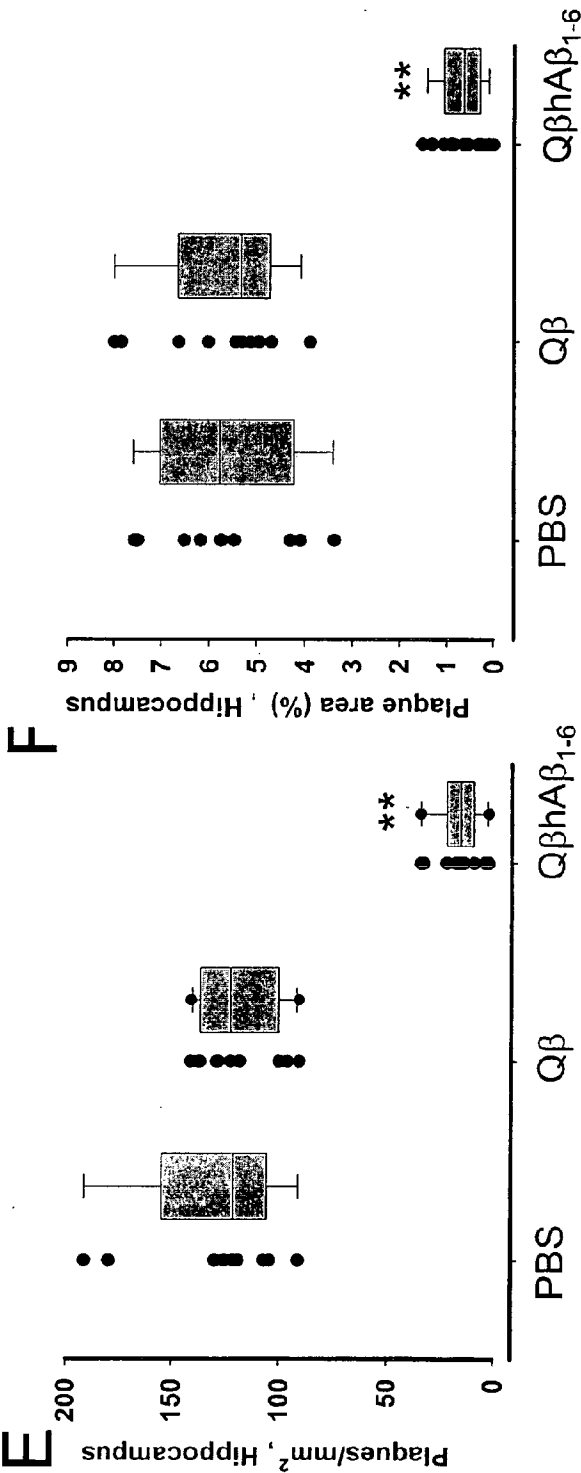


Fig. 12E

Fig. 12F

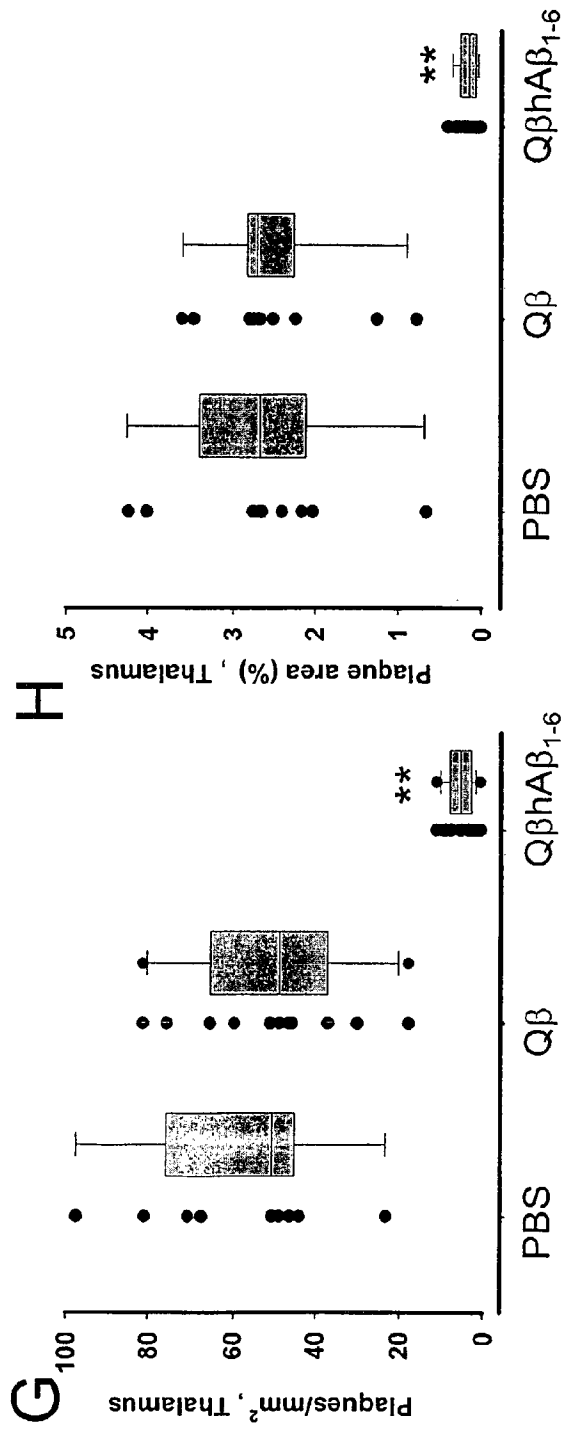


Fig. 12G

Fig. 12H

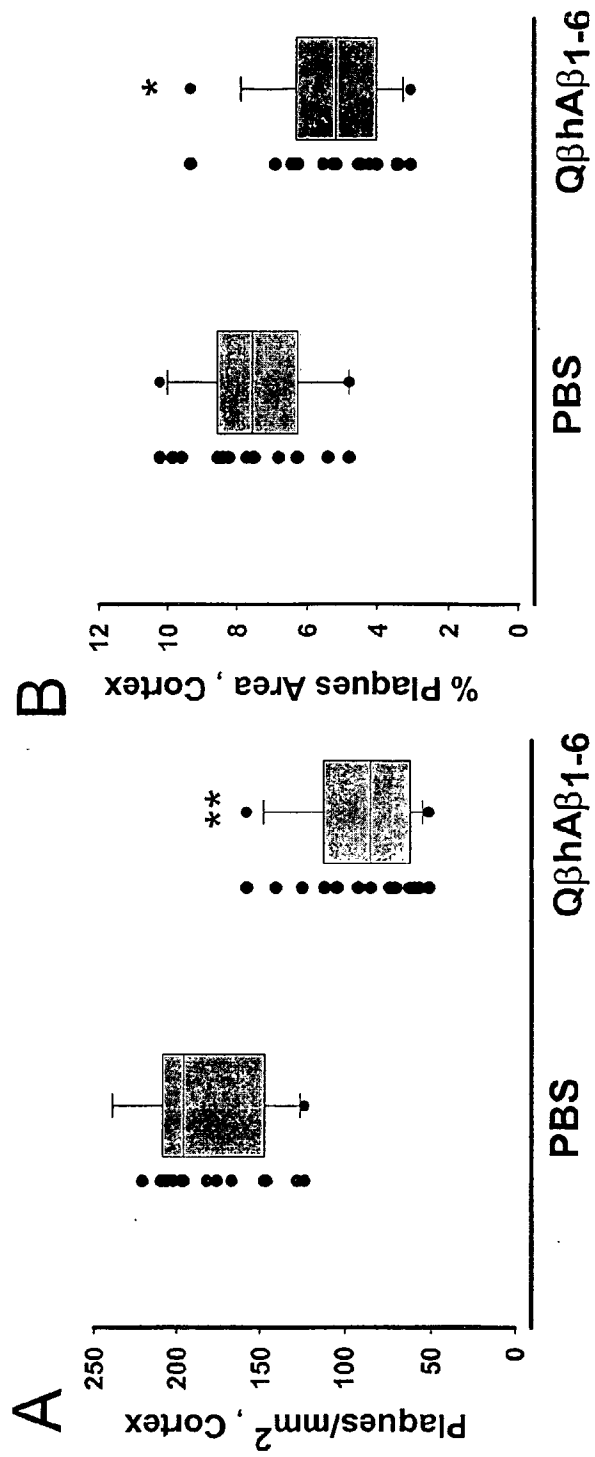


Fig. 13A

Fig. 13B

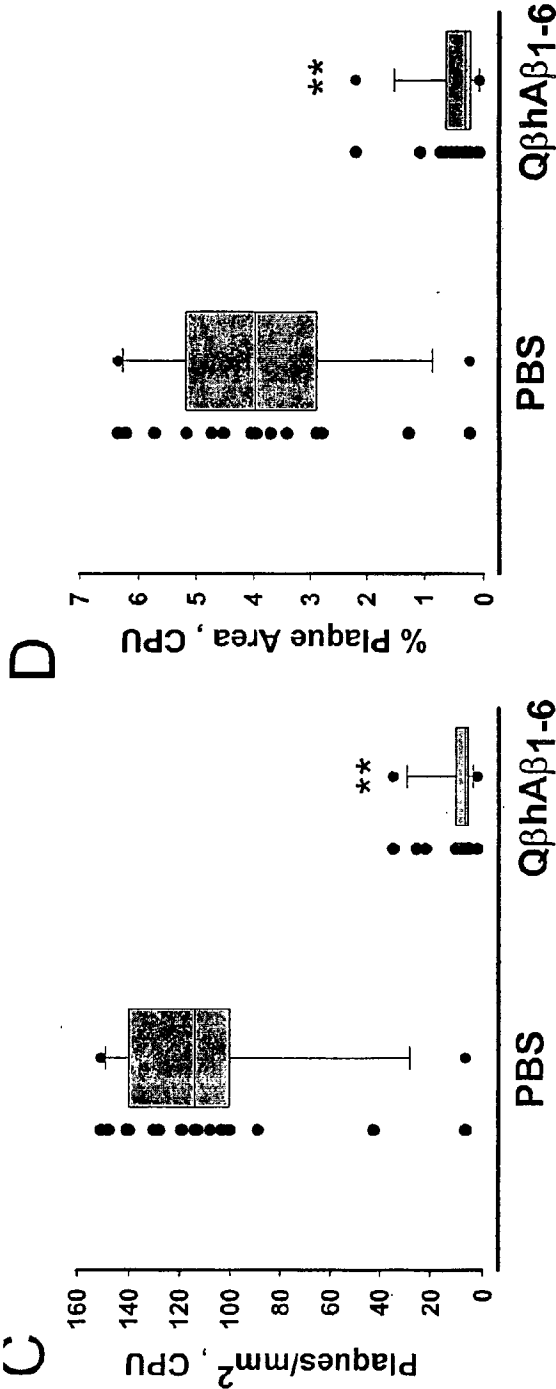


Fig. 13C

Fig. 13D

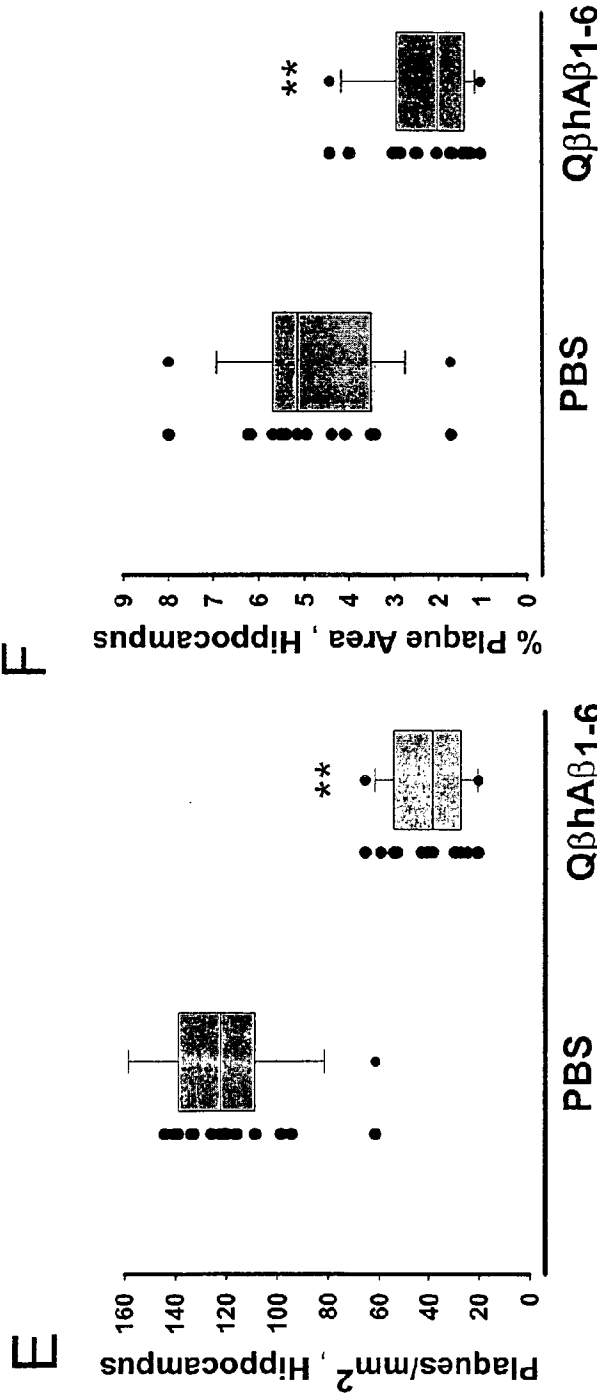


Fig. 13F

Fig. 13E

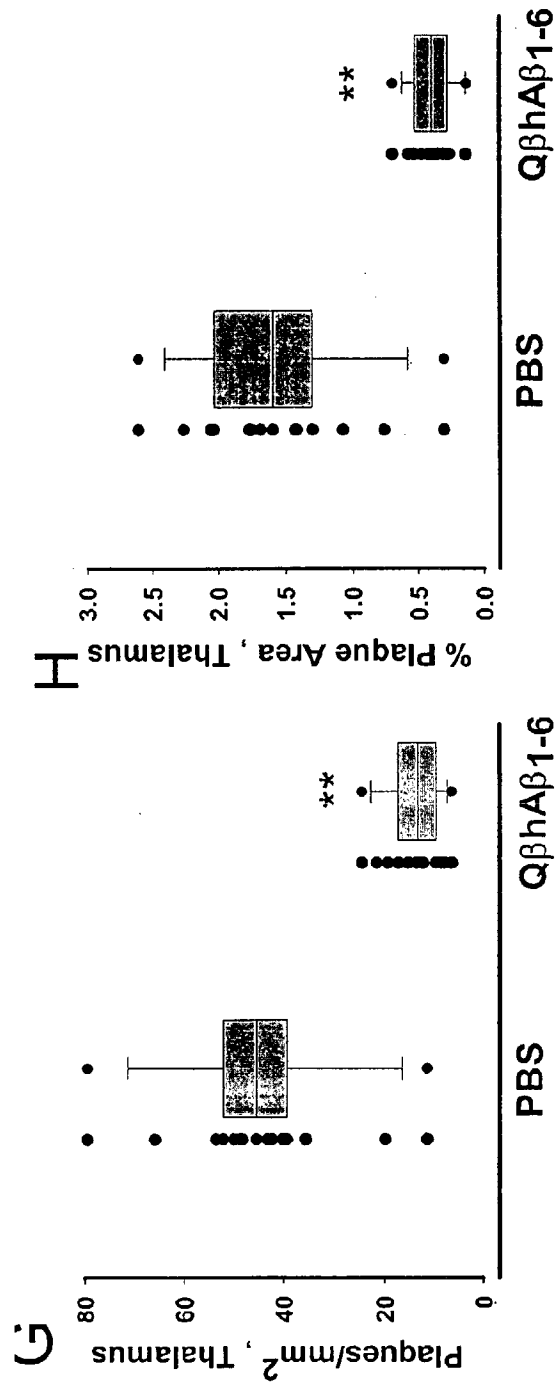


Fig. 13H

Fig. 13G

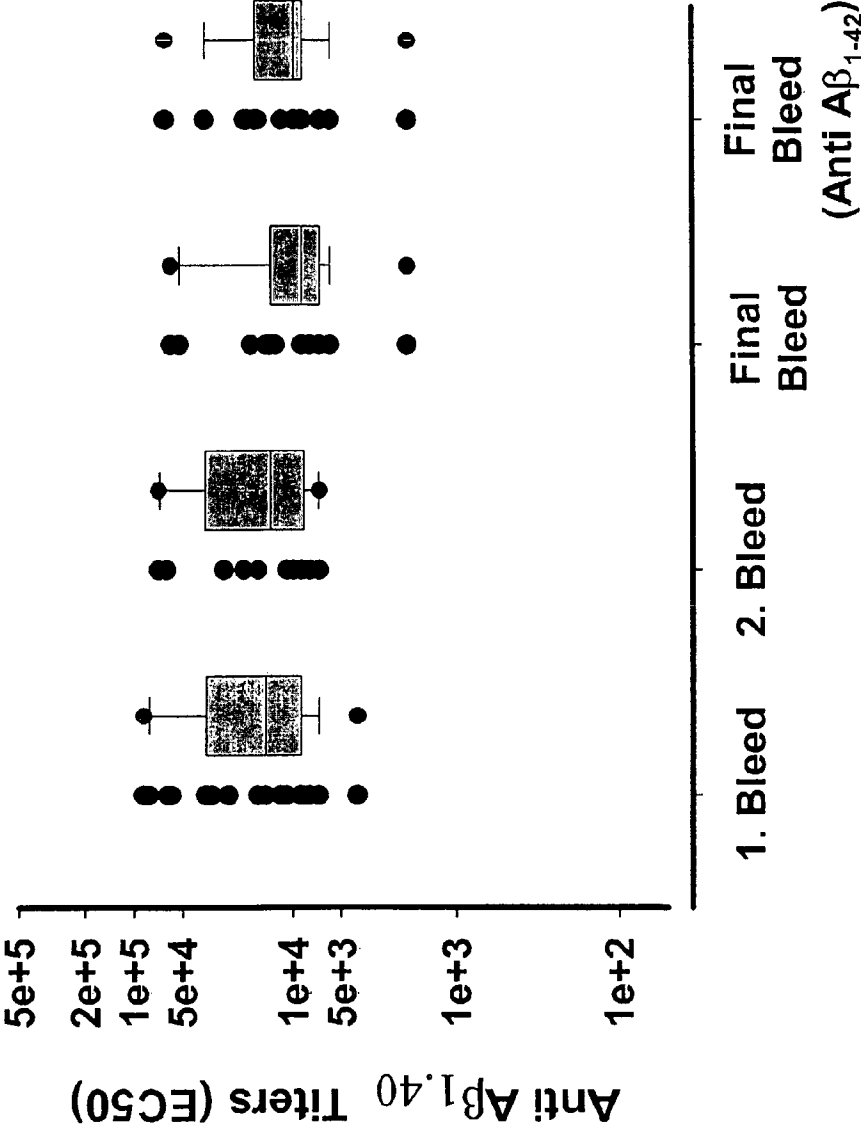


Fig. 14

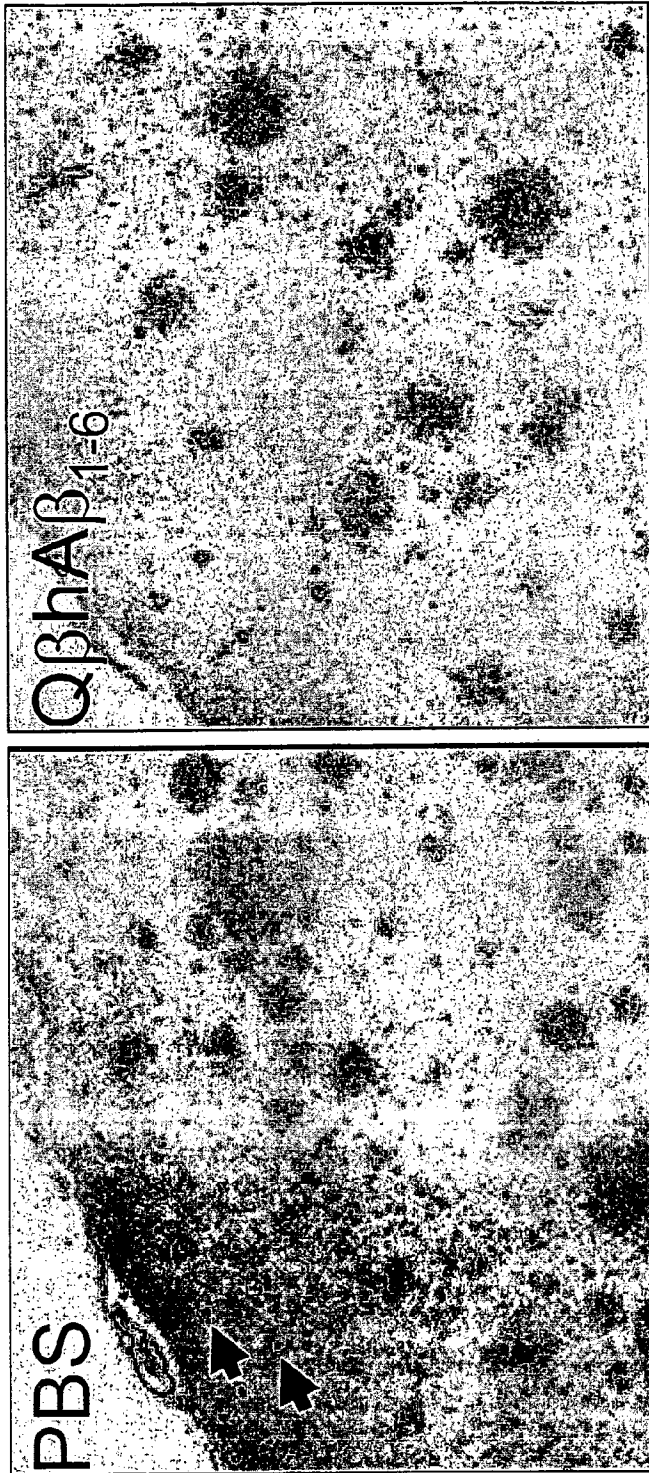


Fig. 15B

Fig. 15A

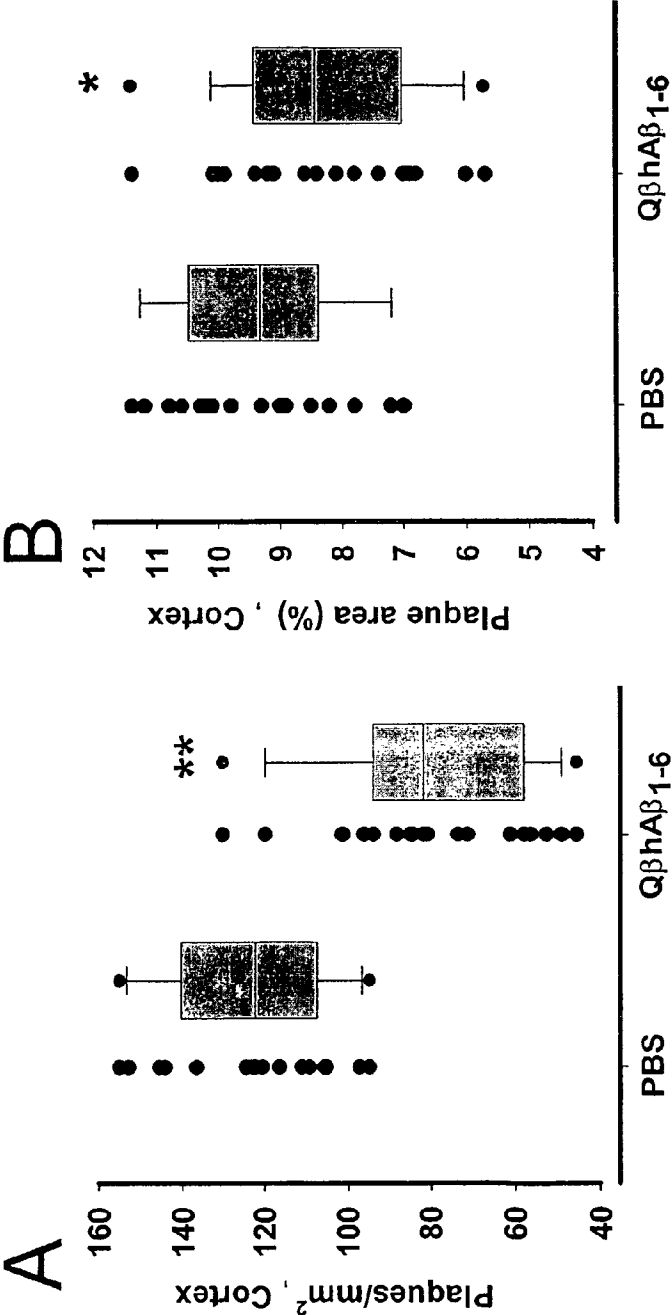


Fig. 16B

Fig. 16A

PA041WO_sequence_listing.ST25.txt
SEQUENCE LISTING

<110> Cytos Biotechnology AG
Novartis Pharma AG
Bachmann, Martin F
Tissot, Alain
Ortmann, Rainer
Lüönd, Rainer
Staufenbiel, Matthias
Frey, Peter

<120> Amyloid Beta 1-6 Antigen Arrays

<130> PA041WO

<150> US 60/396,639

<151> 2002-07-19

<150> US 60/470,432

<151> 2003-05-15

<160> 93

<170> PatentIn version 3.2

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<211> 172

<212> PRT

<213> Escherichia coli

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35 40 45

Ser Lys Ser Phe Leu Ala Asn Asp Gly Gln Ser Lys Pro Met Asn Leu
50 55 60

Asp Ile Glu Leu Val Asn Cys Asp Ile Thr Ala Phe Lys Asn Gly Asn
65 70 75 80

Ala Lys Thr Gly Ser Val Lys Leu Ala Phe Thr Gly Pro Thr Val Ser
85 90 95

Gly His Pro Ser Glu Leu Ala Thr Asn Gly Gly Pro Gly Thr Ala Ile
100 105 110

Met Ile Gln Ala Ala Gly Lys Asn Val Pro Phe Asp Gly Thr Glu Gly
115 120 125

Asp Pro Asn Leu Leu Lys Asp Gly Asp Asn Val Leu His Tyr Thr Thr
130 135 140

PA041WO_sequence_listing.ST25.txt
 Val Gly Lys Lys Ser Ser Asp Gly Asn Ala Gln Ile Thr Glu Gly Ala
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Phe Ser Gly Val Ala Thr Phe Asn Leu Ser Tyr Gln
 165 170

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 <211> 182
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Ser Ser Thr Ala Ala Leu Ala Ala Ala Thr Thr Val Asn Gly Gly Thr
 20 25 30

Val His Phe Lys Gly Glu Val Val Asn Ala Ala Cys Ala Val Asp Ala
 35 40 45

Gly Ser Val Asp Gln Thr Val Gln Leu Gly Gln Val Arg Thr Ala Ser
 50 55 60

Leu Ala Gln Glu Gly Ala Thr Ser Ser Ala Val Gly Phe Asn Ile Gln
 65 70 75 80

Leu Asn Asp Cys Asp Thr Asn Val Ala Ser Lys Ala Ala Val Ala Phe
 85 90 95

Leu Gly Thr Ala Ile Asp Ala Gly His Thr Asn Val Leu Ala Leu Gln
 100 105 110

Ser Ser Ala Ala Gly Ser Ala Thr Asn Val Gly Val Gln Ile Leu Asp
 115 120 125

Arg Thr Gly Ala Ala Leu Thr Leu Asp Gly Ala Thr Phe Ser Ser Glu
 130 135 140

Thr Thr Leu Asn Asn Gly Thr Asn Thr Ile Pro Phe Gln Ala Arg Tyr
 145 150 155 160

Phe Ala Thr Gly Ala Ala Thr Pro Gly Ala Ala Asn Ala Asp Ala Thr
 165 170 175

Phe Lys Val Gln Tyr Gln
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<210> 3
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 <212> DNA
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gtttgtccgc gatgctttac ctctatgagt caaaatggcc ccaatgtttc atcttttggg 180
ggaaactgtg cagtgttggc agtcaaactc gttgacaaac aaagtgtaca gaacgactgc 240
ccatgtcgat ttagaaatag ttttttgaaa ggaaagcagc atgaaaatta aaactctggc 300
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tgttgcatct aaagccgctg ttgccttttt aggtacggcg attgatgcgg gtcataccaa 600
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ggacagaacg ggtgctgcgc tgacgctgga tgggtgcgaca tttagttcag aaacaaccct 720
gaataacgga accaatacca ttccgttcca ggcgcgttat ttgcaaccg gggccgcaac 780
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tcagggacgt tca 853

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<213> Bacteriophage Q-beta

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<400> 4

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Gln Thr Leu Val Leu Asn Pro Arg Gly Val Asn Pro Thr Asn Gly Val
20          25          30

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```

Ala Ser Leu Ser Gln Ala Gly Ala Val Pro Ala Leu Glu Lys Arg Val
35          40          45

```

```

Thr Val Ser Val Ser Gln Pro Ser Arg Asn Arg Lys Asn Tyr Lys Val
50          55          60

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```

Gln Val Lys Ile Gln Asn Pro Thr Ala Cys Thr Ala Asn Gly Ser Cys
65          70          75          80

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Asp Pro Ser Val Thr Arg Gln Ala Tyr Ala Asp Val Thr Phe Ser Phe
85          90          95

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Thr Gln Tyr Ser Thr Asp Glu Glu Arg Ala Phe Val Arg Thr Glu Leu
100         105         110

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Ala Ala Leu Leu Ala Ser Pro Leu Leu Ile Asp Ala Ile Asp Gln Leu
3

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PA041WO_sequence_listing.ST25.txt

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120

125

Asn Pro Ala Tyr
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20 25 30Val Ala Ser Leu Ser Gln Ala Gly Ala Val Pro Ala Leu Glu Lys Arg
35 40 45Val Thr Val Ser Val Ser Gln Pro Ser Arg Asn Arg Lys Asn Tyr Lys
50 55 60Val Gln Val Lys Ile Gln Asn Pro Thr Ala Cys Thr Ala Asn Gly Ser
65 70 75 80Cys Asp Pro Ser Val Thr Arg Gln Ala Tyr Ala Asp Val Thr Phe Ser
85 90 95Phe Thr Gln Tyr Ser Thr Asp Glu Glu Arg Ala Phe Val Arg Thr Glu
100 105 110Leu Ala Ala Leu Leu Ala Ser Pro Leu Leu Ile Asp Ala Ile Asp Gln
115 120 125Leu Asn Pro Ala Tyr Trp Thr Leu Leu Ile Ala Gly Gly Gly Ser Gly
130 135 140Ser Lys Pro Asp Pro Val Ile Pro Asp Pro Pro Ile Asp Pro Pro Pro
145 150 155 160Gly Thr Gly Lys Tyr Thr Cys Pro Phe Ala Ile Trp Ser Leu Glu Glu
165 170 175Val Tyr Glu Pro Pro Thr Lys Asn Arg Pro Trp Pro Ile Tyr Asn Ala
180 185 190Val Glu Leu Gln Pro Arg Glu Phe Asp Val Ala Leu Lys Asp Leu Leu
195 200 205Gly Asn Thr Lys Trp Arg Asp Trp Asp Ser Arg Leu Ser Tyr Thr Thr
210 215 220

PA041WO_sequence_listing.ST25.txt

Phe Arg Gly Cys Arg Gly Asn Gly Tyr Ile Asp Leu Asp Ala Thr Tyr
225 230 235 240

Leu Ala Thr Asp Gln Ala Met Arg Asp Gln Lys Tyr Asp Ile Arg Glu
245 250 255

Gly Lys Lys Pro Gly Ala Phe Gly Asn Ile Glu Arg Phe Ile Tyr Leu
260 265 270

Lys Ser Ile Asn Ala Tyr Cys Ser Leu Ser Asp Ile Ala Ala Tyr His
275 280 285

Ala Asp Gly Val Ile Val Gly Phe Trp Arg Asp Pro Ser Ser Gly Gly
290 295 300

Ala Ile Pro Phe Asp Phe Thr Lys Phe Asp Lys Thr Lys Cys Pro Ile
305 310 315 320

Gln Ala Val Ile Val Val Pro Arg Ala
325

<210> 6
<211> 129
<212> PRT
<213> Bacteriophage R17

<400> 6

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Asn Val Thr Val Ala Pro Ser Asn Phe Ala Asn Gly Val Ala Glu Trp
20 25 30

Ile Ser Ser Asn Ser Arg Ser Gln Ala Tyr Lys Val Thr Cys Ser Val
35 40 45

Arg Gln Ser Ser Ala Gln Asn Arg Lys Tyr Thr Ile Lys Val Glu Val
50 55 60

Pro Lys Val Ala Thr Gln Thr Val Gly Gly Val Glu Leu Pro Val Ala
65 70 75 80

Ala Trp Arg Ser Tyr Leu Asn Met Glu Leu Thr Ile Pro Ile Phe Ala
85 90 95

Thr Asn Ser Asp Cys Glu Leu Ile Val Lys Ala Met Gln Gly Leu Leu
100 105 110

Lys Asp Gly Asn Pro Ile Pro Ser Ala Ile Ala Ala Asn Ser Gly Ile
115 120 125

PA041WO_sequence_listing.ST25.txt

Tyr

<210> 7
 <211> 130
 <212> PRT
 <213> Bacteriophage fr

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 1 5 10 15

Gly Asp Val Lys Val Ala Pro Ser Asn Phe Ala Asn Gly Val Ala Glu
 20 25 30

Trp Ile Ser Ser Asn Ser Arg Ser Gln Ala Tyr Lys Val Thr Cys Ser
 35 40 45

Val Arg Gln Ser Ser Ala Asn Asn Arg Lys Tyr Thr Val Lys Val Glu
 50 55 60

Val Pro Lys Val Ala Thr Gln Val Gln Gly Gly Val Glu Leu Pro Val
 65 70 75 80

Ala Ala Trp Arg Ser Tyr Met Asn Met Glu Leu Thr Ile Pro Val Phe
 85 90 95

Ala Thr Asn Asp Asp Cys Ala Leu Ile Val Lys Ala Leu Gln Gly Thr
 100 105 110

Phe Lys Thr Gly Asn Pro Ile Ala Thr Ala Ile Ala Ala Asn Ser Gly
 115 120 125

Ile Tyr
 130

<210> 8
 <211> 130
 <212> PRT
 <213> Bacteriophage GA

<400> 8

Met Ala Thr Leu Arg Ser Phe Val Leu Val Asp Asn Gly Gly Thr Gly
 1 5 10 15

Asn Val Thr Val Val Pro Val Ser Asn Ala Asn Gly Val Ala Glu Trp
 20 25 30

Leu Ser Asn Asn Ser Arg Ser Gln Ala Tyr Arg Val Thr Ala Ser Tyr
 35 40 45

Arg Ala Ser Gly Ala Asp Lys Arg Lys Tyr Ala Ile Lys Leu Glu Val
 6

50 PA041WO_sequence_listing.ST25.txt
 55 60
 Pro Lys Ile Val Thr Gln Val Val Asn Gly Val Glu Leu Pro Gly Ser
 65 70 75 80
 Ala Trp Lys Ala Tyr Ala Ser Ile Asp Leu Thr Ile Pro Ile Phe Ala
 85 90 95
 Ala Thr Asp Asp Val Thr Val Ile Ser Lys Ser Leu Ala Gly Leu Phe
 100 105 110
 Lys Val Gly Asn Pro Ile Ala Glu Ala Ile Ser Ser Gln Ser Gly Phe
 115 120 125
 Tyr Ala
 130
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 <212> PRT
 <213> Bacteriophage SP
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 1 5 10 15
 Asp Gln Thr Leu Thr Leu Thr Pro Arg Gly Val Asn Pro Thr Asn Gly
 20 25 30
 Val Ala Ser Leu Ser Glu Ala Gly Ala Val Pro Ala Leu Glu Lys Arg
 35 40 45
 Val Thr Val Ser Val Ala Gln Pro Ser Arg Asn Arg Lys Asn Phe Lys
 50 55 60
 Val Gln Ile Lys Leu Gln Asn Pro Thr Ala Cys Thr Arg Asp Ala Cys
 65 70 75 80
 Asp Pro Ser Val Thr Arg Ser Ala Phe Ala Asp Val Thr Leu Ser Phe
 85 90 95
 Thr Ser Tyr Ser Thr Asp Glu Glu Arg Ala Leu Ile Arg Thr Glu Leu
 100 105 110
 Ala Ala Leu Leu Ala Asp Pro Leu Ile Val Asp Ala Ile Asp Asn Leu
 115 120 125
 Asn Pro Ala Tyr
 130

<210> 10
 <211> 329

PA041WO_sequence_listing.ST25.txt

<212> PRT

<213> Bacteriophage SP

<400> 10

Ala Lys Leu Asn Gln Val Thr Leu Ser Lys Ile Gly Lys Asn Gly Asp
 1 5 10 15

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 20 25 30

Ala Ser Leu Ser Glu Ala Gly Ala Val Pro Ala Leu Glu Lys Arg Val
 35 40 45

Thr Val Ser Val Ala Gln Pro Ser Arg Asn Arg Lys Asn Phe Lys Val
 50 55 60

Gln Ile Lys Leu Gln Asn Pro Thr Ala Cys Thr Arg Asp Ala Cys Asp
 65 70 75 80

Pro Ser Val Thr Arg Ser Ala Phe Ala Asp Val Thr Leu Ser Phe Thr
 85 90 95

Ser Tyr Ser Thr Asp Glu Glu Arg Ala Leu Ile Arg Thr Glu Leu Ala
 100 105 110

Ala Leu Leu Ala Asp Pro Leu Ile Val Asp Ala Ile Asp Asn Leu Asn
 115 120 125

Pro Ala Tyr Trp Ala Ala Leu Leu Val Ala Ser Ser Gly Gly Gly Asp
 130 135 140

Asn Pro Ser Asp Pro Asp Val Pro Val Val Pro Asp Val Lys Pro Pro
 145 150 155 160

Asp Gly Thr Gly Arg Tyr Lys Cys Pro Phe Ala Cys Tyr Arg Leu Gly
 165 170 175

Ser Ile Tyr Glu Val Gly Lys Glu Gly Ser Pro Asp Ile Tyr Glu Arg
 180 185 190

Gly Asp Glu Val Ser Val Thr Phe Asp Tyr Ala Leu Glu Asp Phe Leu
 195 200 205

Gly Asn Thr Asn Trp Arg Asn Trp Asp Gln Arg Leu Ser Asp Tyr Asp
 210 215 220

Ile Ala Asn Arg Arg Arg Cys Arg Gly Asn Gly Tyr Ile Asp Leu Asp
 225 230 235 240

Ala Thr Ala Met Gln Ser Asp Asp Phe Val Leu Ser Gly Arg Tyr Gly
 245 250 255

PA041WO_sequence_listing.ST25.txt

Val Arg Lys Val Lys Phe Pro Gly Ala Phe Gly Ser Ile Lys Tyr Leu
 260 265 270

Leu Asn Ile Gln Gly Asp Ala Trp Leu Asp Leu Ser Glu Val Thr Ala
 275 280 285

Tyr Arg Ser Tyr Gly Met Val Ile Gly Phe Trp Thr Asp Ser Lys Ser
 290 295 300

Pro Gln Leu Pro Thr Asp Phe Thr Gln Phe Asn Ser Ala Asn Cys Pro
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Val Gln Thr Val Ile Ile Ile Pro Ser
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 <211> 130
 <212> PRT
 <213> Bacteriophage MS2

<400> 11

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Gly Asp Val Thr Val Ala Pro Ser Asn Phe Ala Asn Gly Val Ala Glu
 20 25 30

Trp Ile Ser Ser Asn Ser Arg Ser Gln Ala Tyr Lys Val Thr Cys Ser
 35 40 45

Val Arg Gln Ser Ser Ala Gln Asn Arg Lys Tyr Thr Ile Lys Val Glu
 50 55 60

Val Pro Lys Val Ala Thr Gln Thr Val Gly Gly Val Glu Leu Pro Val
 65 70 75 80

Ala Ala Trp Arg Ser Tyr Leu Asn Met Glu Leu Thr Ile Pro Ile Phe
 85 90 95

Ala Thr Asn Ser Asp Cys Glu Leu Ile Val Lys Ala Met Gln Gly Leu
 100 105 110

Leu Lys Asp Gly Asn Pro Ile Pro Ser Ala Ile Ala Ala Asn Ser Gly
 115 120 125

Ile Tyr
 130

<210> 12
 <211> 133
 <212> PRT
 <213> Bacteriophage M11

PA041WO_sequence_listing.ST25.txt

<400> 12

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Asp Val Thr Leu Asp Leu Asn Pro Arg Gly Val Asn Pro Thr Asn Gly
 20 25 30

Val Ala Ala Leu Ser Glu Ala Gly Ala Val Pro Ala Leu Glu Lys Arg
 35 40 45

Val Thr Ile Ser Val Ser Gln Pro Ser Arg Asn Arg Lys Asn Tyr Lys
 50 55 60

Val Gln Val Lys Ile Gln Asn Pro Thr Ser Cys Thr Ala Ser Gly Thr
 65 70 75 80

Cys Asp Pro Ser Val Thr Arg Ser Ala Tyr Ser Asp Val Thr Phe Ser
 85 90 95

Phe Thr Gln Tyr Ser Thr Val Glu Glu Arg Ala Leu Val Arg Thr Glu
 100 105 110

Leu Gln Ala Leu Leu Ala Asp Pro Met Leu Val Asn Ala Ile Asp Asn
 115 120 125

Leu Asn Pro Ala Tyr
 130

<210> 13

<211> 133

<212> PRT

<213> Bacteriophage MX1

<400> 13

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Asp Val Thr Leu Asn Leu Asn Pro Arg Gly Val Asn Pro Thr Asn Gly
 20 25 30

Val Ala Ala Leu Ser Glu Ala Gly Ala Val Pro Ala Leu Glu Lys Arg
 35 40 45

Val Thr Ile Ser Val Ser Gln Pro Ser Arg Asn Arg Lys Asn Tyr Lys
 50 55 60

Val Gln Val Lys Ile Gln Asn Pro Thr Ser Cys Thr Ala Ser Gly Thr
 65 70 75 80

Cys Asp Pro Ser Val Thr Arg Ser Ala Tyr Ala Asp Val Thr Phe Ser
 85 90 95

PA041W0_sequence_listing.ST25.txt

Phe Thr Gln Tyr Ser Thr Asp Glu Glu Arg Ala Leu Val Arg Thr Glu
 100 105 110

Leu Lys Ala Leu Leu Ala Asp Pro Met Leu Ile Asp Ala Ile Asp Asn
 115 120 125

Leu Asn Pro Ala Tyr
 130

<210> 14
 <211> 330
 <212> PRT
 <213> Bacteriophage NL95

<400> 14

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Asn Gln Thr Leu Thr Leu Thr Pro Arg Gly Val Asn Pro Thr Asn Gly
 20 25 30

Val Ala Ser Leu Ser Glu Ala Gly Ala Val Pro Ala Leu Glu Lys Arg
 35 40 45

Val Thr Val Ser Val Ala Gln Pro Ser Arg Asn Arg Lys Asn Tyr Lys
 50 55 60

Val Gln Ile Lys Leu Gln Asn Pro Thr Ala Cys Thr Lys Asp Ala Cys
 65 70 75 80

Asp Pro Ser Val Thr Arg Ser Gly Ser Arg Asp Val Thr Leu Ser Phe
 85 90 95

Thr Ser Tyr Ser Thr Glu Arg Glu Arg Ala Leu Ile Arg Thr Glu Leu
 100 105 110

Ala Ala Leu Leu Lys Asp Asp Leu Ile Val Asp Ala Ile Asp Asn Leu
 115 120 125

Asn Pro Ala Tyr Trp Ala Ala Leu Leu Ala Ala Ser Pro Gly Gly Gly
 130 135 140

Asn Asn Pro Tyr Pro Gly Val Pro Asp Ser Pro Asn Val Lys Pro Pro
 145 150 155 160

Gly Gly Thr Gly Thr Tyr Arg Cys Pro Phe Ala Cys Tyr Arg Arg Gly
 165 170 175

Glu Leu Ile Thr Glu Ala Lys Asp Gly Ala Cys Ala Leu Tyr Ala Cys
 180 185 190

PA041WO_sequence_listing.ST25.txt

Gly Ser Glu Ala Leu Val Glu Phe Glu Tyr Ala Leu Glu Asp Phe Leu
 195 200 205

Gly Asn Glu Phe Trp Arg Asn Trp Asp Gly Arg Leu Ser Lys Tyr Asp
 210 215 220

Ile Glu Thr His Arg Arg Cys Arg Gly Asn Gly Tyr Val Asp Leu Asp
 225 230 235 240

Ala Ser Val Met Gln Ser Asp Glu Tyr Val Leu Ser Gly Ala Tyr Asp
 245 250 255

Val Val Lys Met Gln Pro Pro Gly Thr Phe Asp Ser Pro Arg Tyr Tyr
 260 265 270

Leu His Leu Met Asp Gly Ile Tyr Val Asp Leu Ala Glu Val Thr Ala
 275 280 285

Tyr Arg Ser Tyr Gly Met Val Ile Gly Phe Trp Thr Asp Ser Lys Ser
 290 295 300

Pro Gln Leu Pro Thr Asp Phe Thr Arg Phe Asn Arg His Asn Cys Pro
 305 310 315 320

Val Gln Thr Val Ile Val Ile Pro Ser Leu
 325 330

<210> 15
 <211> 129
 <212> PRT
 <213> Bacteriophage f2

<400> 15

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Asn Val Thr Val Ala Pro Ser Asn Phe Ala Asn Gly Val Ala Glu Trp
 20 25 30

Ile Ser Ser Asn Ser Arg Ser Gln Ala Tyr Lys Val Thr Cys Ser Val
 35 40 45

Arg Gln Ser Ser Ala Gln Asn Arg Lys Tyr Thr Ile Lys Val Glu Val
 50 55 60

Pro Lys Val Ala Thr Gln Thr Val Gly Gly Val Glu Leu Pro Val Ala
 65 70 75 80

Ala Trp Arg Ser Tyr Leu Asn Leu Glu Leu Thr Ile Pro Ile Phe Ala
 85 90 95

PA041WO_sequence_listing.ST25.txt

Thr Asn Ser Asp Cys Glu Leu Ile Val Lys Ala Met Gln Gly Leu Leu
 100 105 110

Lys Asp Gly Asn Pro Ile Pro Ser Ala Ile Ala Ala Asn Ser Gly Ile
 115 120 125

Tyr

<210> 16
 <211> 128
 <212> PRT
 <213> Bacteriophage PP7

<400> 16

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 1 5 10 15

Thr Glu Ile Gln Ser Thr Ala Asp Arg Gln Ile Phe Glu Glu Lys Val
 20 25 30

Gly Pro Leu Val Gly Arg Leu Arg Leu Thr Ala Ser Leu Arg Gln Asn
 35 40 45

Gly Ala Lys Thr Ala Tyr Arg Val Asn Leu Lys Leu Asp Gln Ala Asp
 50 55 60

Val Val Asp Cys Ser Thr Ser Val Cys Gly Glu Leu Pro Lys Val Arg
 65 70 75 80

Tyr Thr Gln Val Trp Ser His Asp Val Thr Ile Val Ala Asn Ser Thr
 85 90 95

Glu Ala Ser Arg Lys Ser Leu Tyr Asp Leu Thr Lys Ser Leu Val Ala
 100 105 110

Thr Ser Gln Val Glu Asp Leu Val Val Asn Leu Val Pro Leu Gly Arg
 115 120 125

<210> 17
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 <212> PRT
 <213> Artificial Sequence

<220>
 <223> Bacteriophage Qbeta 240 mutant

<400> 17

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 1 5 10 15

Gln Thr Leu Val Leu Asn Pro Arg Gly Val Asn Pro Thr Asn Gly Val
 20 25 30

PA041w0_sequence_listing.ST25.txt

Ala Ser Leu Ser Gln Ala Gly Ala Val Pro Ala Leu Glu Lys Arg Val
35 40 45

Thr Val Ser Val Ser Gln Pro Ser Arg Asn Arg Lys Asn Tyr Lys Val
50 55 60

Gln Val Lys Ile Gln Asn Pro Thr Ala Cys Thr Ala Asn Gly Ser Cys
65 70 75 80

Asp Pro Ser Val Thr Arg Gln Lys Tyr Ala Asp Val Thr Phe Ser Phe
85 90 95

Thr Gln Tyr Ser Thr Asp Glu Glu Arg Ala Phe Val Arg Thr Glu Leu
100 105 110

Ala Ala Leu Leu Ala Ser Pro Leu Leu Ile Asp Ala Ile Asp Gln Leu
115 120 125

Asn Pro Ala Tyr
130

<210>	18
<211>	132
<212>	PRT
<213>	Artificial Sequence

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<220>
<223> Bacteriophage Q-beta 243 mutant
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<400> 18

Ala Lys Leu Glu Thr Val Thr Leu Gly Lys Ile Gly Lys Asp Gly Lys
1 5 10 15

Gln Thr Leu Val Leu Asn Pro Arg Gly Val Asn Pro Thr Asn Gly Val
20 25 30

Ala Ser Leu Ser Gln Ala Gly Ala Val Pro Ala Leu Glu Lys Arg Val
35 40 45

Thr Val Ser Val Ser Gln Pro Ser Arg Asn Arg Lys Asn Tyr Lys Val
50 55 60

Gln Val Lys Ile Gln Asn Pro Thr Ala Cys Thr Ala Asn Gly Ser Cys
65 70 75 80

Asp Pro Ser Val Thr Arg Gln Lys Tyr Ala Asp Val Thr Phe Ser Phe
85 90 95

Thr Gln Tyr Ser Thr Asp Glu Glu Arg Ala Phe Val Arg Thr Glu Leu
100 105 110

Ala Ala Leu Leu Ala Ser Pro Leu Leu Ile Asp Ala Ile Asp Gln Leu
14

PA041WO_sequence_listing.ST25.txt

115

120

125

Asn Pro Ala Tyr
130

<210> 19
<211> 132
<212> PRT
<213> Artificial Sequence

<220>
<223> Bacteriophage Q-beta 250 mutant

<400> 19

Ala Arg Leu Glu Thr Val Thr Leu Gly Asn Ile Gly Arg Asp Gly Lys
1 5 10 15

Gln Thr Leu Val Leu Asn Pro Arg Gly Val Asn Pro Thr Asn Gly Val
20 25 30

Ala Ser Leu Ser Gln Ala Gly Ala Val Pro Ala Leu Glu Lys Arg Val
35 40 45

Thr Val Ser Val Ser Gln Pro Ser Arg Asn Arg Lys Asn Tyr Lys Val
50 55 60

Gln Val Lys Ile Gln Asn Pro Thr Ala Cys Thr Ala Asn Gly Ser Cys
65 70 75 80

Asp Pro Ser Val Thr Arg Gln Lys Tyr Ala Asp Val Thr Phe Ser Phe
85 90 95

Thr Gln Tyr Ser Thr Asp Glu Glu Arg Ala Phe Val Arg Thr Glu Leu
100 105 110

Ala Ala Leu Leu Ala Ser Pro Leu Leu Ile Asp Ala Ile Asp Gln Leu
115 120 125

Asn Pro Ala Tyr
130

<210> 20
<211> 132
<212> PRT
<213> Artificial Sequence

<220>
<223> Bacteriophage Q-beta 251 mutant

<400> 20

Ala Lys Leu Glu Thr Val Thr Leu Gly Asn Ile Gly Lys Asp Gly Arg
1 5 10 15

Gln Thr Leu Val Leu Asn Pro Arg Gly Val Asn Pro Thr Asn Gly Val
15

PA041WO_sequence_listing.ST25.txt

20

25

30

Ala Ser Leu Ser Gln Ala Gly Ala Val Pro Ala Leu Glu Lys Arg Val
 35 40 45

Thr Val Ser Val Ser Gln Pro Ser Arg Asn Arg Lys Asn Tyr Lys Val
 50 55 60

Gln Val Lys Ile Gln Asn Pro Thr Ala Cys Thr Ala Asn Gly Ser Cys
 65 70 75 80

Asp Pro Ser Val Thr Arg Gln Lys Tyr Ala Asp Val Thr Phe Ser Phe
 85 90 95

Thr Gln Tyr Ser Thr Asp Glu Glu Arg Ala Phe Val Arg Thr Glu Leu
 100 105 110

Ala Ala Leu Leu Ala Ser Pro Leu Leu Ile Asp Ala Ile Asp Gln Leu
 115 120 125

Asn Pro Ala Tyr
 130

<210> 21
 <211> 132
 <212> PRT
 <213> Artificial Sequence

<220>
 <223> Bacteriophage Q-beta 259 mutant

<400> 21

Ala Arg Leu Glu Thr Val Thr Leu Gly Asn Ile Gly Lys Asp Gly Arg
 1 5 10 15

Gln Thr Leu Val Leu Asn Pro Arg Gly Val Asn Pro Thr Asn Gly Val
 20 25 30

Ala Ser Leu Ser Gln Ala Gly Ala Val Pro Ala Leu Glu Lys Arg Val
 35 40 45

Thr Val Ser Val Ser Gln Pro Ser Arg Asn Arg Lys Asn Tyr Lys Val
 50 55 60

Gln Val Lys Ile Gln Asn Pro Thr Ala Cys Thr Ala Asn Gly Ser Cys
 65 70 75 80

Asp Pro Ser Val Thr Arg Gln Lys Tyr Ala Asp Val Thr Phe Ser Phe
 85 90 95

Thr Gln Tyr Ser Thr Asp Glu Glu Arg Ala Phe Val Arg Thr Glu Leu
 100 105 110

PA041WO_sequence_listing.ST25.txt

Ala Ala Leu Leu Ala Ser Pro Leu Leu Ile Asp Ala Ile Asp Gln Leu
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Asn Pro Ala Tyr
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<210> 22
 <211> 185
 <212> PRT
 <213> Hepatitis B virus
 <400> 22

Met Asp Ile Asp Pro Tyr Lys Glu Phe Gly Ala Thr Val Glu Leu Leu
 1 5 10 15

Ser Phe Leu Pro Ser Asp Phe Phe Pro Ser Val Arg Asp Leu Leu Asp
 20 25 30

Thr Ala Ser Ala Leu Tyr Arg Glu Ala Leu Glu Ser Pro Glu His Cys
 35 40 45

Ser Pro His His Thr Ala Leu Arg Gln Ala Ile Leu Cys Trp Gly Glu
 50 55 60

Leu Met Thr Leu Ala Thr Trp Val Gly Asn Asn Leu Glu Asp Pro Ala
 65 70 75 80

Ser Arg Asp Leu Val Val Asn Tyr Val Asn Thr Asn Met Gly Leu Lys
 85 90 95

Ile Arg Gln Leu Leu Trp Phe His Ile Ser Cys Leu Thr Phe Gly Arg
 100 105 110

Glu Thr Val Leu Glu Tyr Leu Val Ser Phe Gly Val Trp Ile Arg Thr
 115 120 125

Pro Pro Ala Tyr Arg Pro Pro Asn Ala Pro Ile Leu Ser Thr Leu Pro
 130 135 140

Glu Thr Thr Val Val Arg Arg Arg Asp Arg Gly Arg Ser Pro Arg Arg
 145 150 155 160

Arg Thr Pro Ser Pro Arg Arg Arg Arg Ser Gln Ser Pro Arg Arg Arg
 165 170 175

Arg Ser Gln Ser Arg Glu Ser Gln Cys
 180 185

<210> 23
 <211> 212
 <212> PRT
 <213> Hepatitis B virus

PA041WO_sequence_listing.ST25.txt

<400> 23

Met Gln Leu Phe His Leu Cys Leu Ile Ile Ser Cys Ser Cys Pro Thr
 1 5 10 15

Val Gln Ala Ser Lys Leu Cys Leu Gly Trp Leu Trp Gly Met Asp Ile
 20 25 30

Asp Pro Tyr Lys Glu Phe Gly Ala Thr Val Glu Leu Leu Ser Phe Leu
 35 40 45

Pro Ser Asp Phe Phe Pro Ser Val Arg Asp Leu Leu Asp Thr Ala Ser
 50 55 60

Ala Leu Tyr Arg Glu Ala Leu Glu Ser Pro Glu His Cys Ser Pro His
 65 70 75 80

His Thr Ala Leu Arg Gln Ala Ile Leu Cys Trp Gly Asp Leu Met Asn
 85 90 95

Leu Ala Thr Trp Val Gly Gly Asn Leu Glu Asp Pro Val Ser Arg Asp
 100 105 110

Leu Val Val Gly Tyr Val Asn Thr Thr Val Gly Leu Lys Phe Arg Gln
 115 120 125

Leu Leu Trp Phe His Ile Ser Cys Leu Thr Phe Gly Arg Glu Thr Val
 130 135 140

Ile Glu Tyr Leu Val Ser Phe Gly Val Trp Ile Arg Thr Pro Pro Ala
 145 150 155 160

Tyr Arg Pro Pro Asn Ala Pro Ile Leu Ser Thr Leu Pro Glu Thr Thr
 165 170 175

Val Val Arg Arg Arg Gly Arg Ser Pro Arg Arg Arg Thr Pro Ser Pro
 180 185 190

Arg Arg Arg Arg Ser Gln Ser Pro Arg Arg Arg Arg Ser Gln Ser Arg
 195 200 205

Glu Ser Gln Cys
 210

<210> 24

<211> 188

<212> PRT

<213> Hepatitis B virus

<400> 24

Met Asp Ile Asp Pro Tyr Lys Glu Phe Gly Ser Ser Tyr Gln Leu Leu
 1 5 10 15

PA041WO_sequence_listing.ST25.txt

Asn Phe Leu Pro Leu Asp Phe Phe Pro Asp Leu Asn Ala Leu Val Asp
 20 25 30
 Thr Ala Thr Ala Leu Tyr Glu Glu Glu Leu Thr Gly Arg Glu His Cys
 35 40 45
 Ser Pro His His Thr Ala Ile Arg Gln Ala Leu Val Cys Trp Asp Glu
 50 55 60
 Leu Thr Lys Leu Ile Ala Trp Met Ser Ser Asn Ile Thr Ser Glu Gln
 65 70 75 80
 Val Arg Thr Ile Ile Val Asn His Val Asn Asp Thr Trp Gly Leu Lys
 85 90 95
 Val Arg Gln Ser Leu Trp Phe His Leu Ser Cys Leu Thr Phe Gly Gln
 100 105 110
 His Thr Val Gln Glu Phe Leu Val Ser Phe Gly Val Trp Ile Arg Thr
 115 120 125
 Pro Ala Pro Tyr Arg Pro Pro Asn Ala Pro Ile Leu Ser Thr Leu Pro
 130 135 140
 Glu His Thr Val Ile Arg Arg Arg Gly Gly Ala Arg Ala Ser Arg Ser
 145 150 155 160
 Pro Arg Arg Arg Thr Pro Ser Pro Arg Arg Arg Arg Ser Gln Ser Pro
 165 170 175
 Arg Arg Arg Arg Ser Gln Ser Pro Ser Thr Asn Cys
 180 185

<210> 25
 <211> 185
 <212> PRT
 <213> Hepatitis B virus

<400> 25

Met Asp Ile Asp Pro Tyr Lys Glu Phe Gly Ala Thr Val Glu Leu Leu
 1 5 10 15
 Ser Phe Leu Pro Ser Asp Phe Phe Pro Ser Val Arg Asp Leu Leu Asp
 20 25 30
 Thr Ala Ser Ala Leu Tyr Arg Glu Ala Leu Glu Ser Pro Glu His Cys
 35 40 45
 Ser Pro His His Thr Ala Leu Arg Gln Ala Ile Leu Cys Trp Gly Glu
 50 55 60

PA041WO_sequence_listing.ST25.txt

Leu Met Thr Leu Ala Thr Trp Val Gly Asn Asn Leu Glu Asp Pro Ala
65 70 75 80

Ser Arg Asp Leu Val Val Asn Tyr Val Asn Thr Asn Met Gly Leu Lys
85 90 95

Ile Arg Gln Leu Leu Trp Phe His Ile Ser Cys Leu Thr Phe Gly Arg
100 105 110

Glu Thr Val Leu Glu Tyr Leu Val Ser Phe Gly Val Trp Ile Arg Thr
115 120 125

Pro Pro Ala Tyr Arg Pro Pro Asn Ala Pro Ile Leu Ser Thr Leu Pro
130 135 140

Glu Thr Thr Val Val Arg Arg Arg Asp Arg Gly Arg Ser Pro Arg Arg
145 150 155 160

Arg Thr Pro Ser Pro Arg Arg Arg Arg Ser Gln Ser Pro Arg Arg Arg
165 170 175

Arg Ser Gln Ser Arg Glu Ser Gln Cys
180 185

<210> 26
<211> 152
<212> PRT
<213> Hepatitis B virus

<400> 26

Met Asp Ile Asp Pro Tyr Lys Glu Phe Gly Ala Thr Val Glu Leu Leu
1 5 10 15

Ser Phe Leu Pro Ser Asp Phe Phe Pro Ser Val Arg Asp Leu Leu Asp
20 25 30

Thr Ala Ala Ala Leu Tyr Arg Asp Ala Leu Glu Ser Pro Glu His Cys
35 40 45

Ser Pro His His Thr Ala Leu Arg Gln Ala Ile Leu Cys Trp Gly Asp
50 55 60

Leu Met Thr Leu Ala Thr Trp Val Gly Thr Asn Leu Glu Asp Gly Gly
65 70 75 80

Lys Gly Gly Ser Arg Asp Leu Val Val Ser Tyr Val Asn Thr Asn Val
85 90 95

Gly Leu Lys Phe Arg Gln Leu Leu Trp Phe His Ile Ser Cys Leu Thr
100 105 110

PA041WO_sequence_listing.ST25.txt

Phe Gly Arg Glu Thr Val Leu Glu Tyr Leu Val Ser Phe Gly Val Trp
 115 120 125

Ile Arg Thr Pro Pro Ala Tyr Arg Pro Pro Asn Ala Pro Ile Leu Ser
 130 135 140

Thr Leu Pro Glu Thr Thr Val Val
 145 150

<210> 27
 <211> 3635
 <212> DNA
 <213> Artificial Sequence

<220>
 <223> plasmid pAP283-58

<400> 27
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 ggaaaatcac atggcaaata agccaatgca accgatcaca tctacagcaa ataaaattgt 180
 gtggtcggat ccaactcgtt tatcaactac attttcagca agtctgttac gccaacgtgt 240
 taaagttggt atagccgaac tgaataatgt ttcagggtcaa tatgtatctg ttataagcg 300
 tcctgcacct aaaccggaag gttgtgcaga tgctgtgtc attatgccga atgaaaacca 360
 atccattcgc acagtgattt cagggtcagc cgaaaacttg gctaccttaa aagcagaatg 420
 ggaaactcac aaacgtaacg ttgacacact cttcgcgagc ggcaacgccg gtttggttt 480
 ctttgacct actgcggcta tcgtatcgtc tgatactact gcttaagctt gtattctata 540
 gtgtcaccta aatcgtatgt gtatgataca taaggttatg tattaattgt agccgcgttc 600
 taacgacaat atgtacaagc ctaattgtgt agcatctggc ttactgaagc agaccctatc 660
 atctctctcg taaactgccg tcagagtcgg tttggttggc cgaaccttct gagtttctgg 720
 taacgccgtt ccgcaccccg gaaatggcca ccgaaccaat cagcagggtc atcgctagcc 780
 agatcctcta cgccggagc atcgtggccg gcatcaccgg cgcacacagt gcggttgctg 840
 gcgcctatat cgccgacatc accgatggg aagatcgggc tcgccacttc gggctcatga 900
 gcgcttgttt cggcgtgggt atggtggcag gccccgtggc cgggggactg ttgggcgcca 960
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 caatctgctc tgatgccgca tagttaagcc aactccgcta tcgctacgtg actgggtcat 1140
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 ggcacccgt tacagacaag ctgtgaccgt ctccgggagc tgcattgtgc agaggttttc 1260
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 tttttatagg ttaatgtcat gataataatg gtttcttaga cgtcagggtg cacttttcgg 1380
 ggaaatgtgc gcggaacccc tttttgttta tttttctaaa tacattcaaa tatgtatccg 1440

PA041WO_sequence_listing.ST25.txt

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gctcaccag aaacgctggt gaaagtaaaa gatgctgaag atcagttggg tgcacgagt	1620
ggttacatcg aactggatct caacagcggg aagatccttg agagttttcg cccgaagaa	1680
cgttttccaa tgatgagcac ttttaaagtt ctgctatgtg gcgcggtatt atcccgtatt	1740
gacgccgggc aagagcaact cggtcgccgc atacactatt ctcagaatga cttggttgag	1800
tactcaccag tcacagaaaa gcatcttacg gatggcatga cagtaagaga attatgcagt	1860
gctgccataa ccatgagtga taacactgcg gccaaacttac ttctgacaac gatcggagga	1920
ccgaaggagc taaccgcttt ttgacacaac atgggggatc atgtaactcg ccttgatcgt	1980
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gcaatggcaa caacgttgcg caaactatta actggcgaac tacttactct agcttcccg	2100
caacaattaa tagactggat ggaggcggat aaagttgcag gaccacttct gcgctcggcc	2160
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attaagcatt ggtaactgtc agaccaagtt tactcatata tacttttagat tgatttaaaa	2400
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gctgctgcca gtggcgataa gtcgtgtctt accgggttg actcaagacg atagttaccg	2820
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acgacctaca ccgaactgag atacctacag cgcgagcatt gagaaagcgc cacgcttccc	2940
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tggtcggtag tcgccagggt gccgacgcgc atctcgactg catggtgcac caatgcttct	3420
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PA041WO_sequence_listing.ST25.txt

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 tacgcaagtt cacgtaaaaa gggatcgcg gaatt 3635

<210> 28
 <211> 131
 <212> PRT
 <213> Bacteriophage AP205

<400> 28

Met Ala Asn Lys Pro Met Gln Pro Ile Thr Ser Thr Ala Asn Lys Ile
 1 5 10 15

Val Trp Ser Asp Pro Thr Arg Leu Ser Thr Thr Phe Ser Ala Ser Leu
 20 25 30

Leu Arg Gln Arg Val Lys Val Gly Ile Ala Glu Leu Asn Asn Val Ser
 35 40 45

Gly Gln Tyr Val Ser Val Tyr Lys Arg Pro Ala Pro Lys Pro Glu Gly
 50 55 60

Cys Ala Asp Ala Cys Val Ile Met Pro Asn Glu Asn Gln Ser Ile Arg
 65 70 75 80

Thr Val Ile Ser Gly Ser Ala Glu Asn Leu Ala Thr Leu Lys Ala Glu
 85 90 95

Trp Glu Thr His Lys Arg Asn Val Asp Thr Leu Phe Ala Ser Gly Asn
 100 105 110

Ala Gly Leu Gly Phe Leu Asp Pro Thr Ala Ala Ile Val Ser Ser Asp
 115 120 125

Thr Thr Ala
 130

<210> 29
 <211> 131
 <212> PRT
 <213> Artificial Sequence

<220>
 <223> AP205 coat protein

<400> 29

Met Ala Asn Lys Thr Met Gln Pro Ile Thr Ser Thr Ala Asn Lys Ile
 1 5 10 15

Val Trp Ser Asp Pro Thr Arg Leu Ser Thr Thr Phe Ser Ala Ser Leu
 20 25 30

PA041WO_sequence_listing.ST25.txt

Leu Arg Gln Arg Val Lys Val Gly Ile Ala Glu Leu Asn Asn Val Ser
 35 40 45

Gly Gln Tyr Val Ser Val Tyr Lys Arg Pro Ala Pro Lys Pro Glu Gly
 50 55 60

Cys Ala Asp Ala Cys Val Ile Met Pro Asn Glu Asn Gln Ser Ile Arg
 65 70 75 80

Thr Val Ile Ser Gly Ser Ala Glu Asn Leu Ala Thr Leu Lys Ala Glu
 85 90 95

Trp Glu Thr His Lys Arg Asn Val Asp Thr Leu Phe Ala Ser Gly Asn
 100 105 110

Ala Gly Leu Gly Phe Leu Asp Pro Thr Ala Ala Ile Val Ser Ser Asp
 115 120 125

Thr Thr Ala
 130

<210> 30
 <211> 3607
 <212> DNA
 <213> Artificial Sequence

<220>
 <223> plasmid pAP281-32

<400> 30
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 acaatgcaac cgatcacatc tacagcaaat aaaattgtgt ggtcggatcc aactcgttta 180
 tcaactacat tttcagcaag tctgttacgc caacgtgtta aagttggtat agccgaactg 240
 aataatgttt cagggtcaata tgtatctgtt tataagcgtc ctgcacctaa accgaaggtc 300
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 agccgaaaac ttggctacct taaaagcaga atgggaaact cacaacgta acgttgacac 420
 actcttcgcg agcggcaacg ccggtttggg tttccttgac cctactgcgg ctatcgtatc 480
 gtctgatact actgcttaag cttgtattct atagtgtcac ctaaatcgta tgtgtatgat 540
 acataagggt atgtattaat ggtagccgcg ttctaacgac aatatgtaca agcctaattg 600
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 cggttggggt ggacagacct ctgagtttct ggtaacgccc ttccgcaccc cggaaatggt 720
 caccgaacca ttcagcaggg tcatcgctag ccagatcctc tacgccggac gcatcgtggc 780
 ccgcatcacc ggcgccacag gtgcggtgct ggcgcctata tcgccgacat caccgatggg 840
 gaagatcggg ctgcgcactt cgggctcatg atcgctgggt tccgcctggg tatggtggca 900

PA041WO_sequence_Listing.ST25.txt

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gggagagcgt	cgatatggtg	cactctcagt	acaatctgct	ctgatgccgc	atagttaagc	1080
caactccgct	atcgctacgt	gactgggtca	tggtgcgcc	ccgacaccgc	ccaacaccgc	1140
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atTTTTctaa	atacattcaa	atatgtatcc	gtcatgaga	caataaccct	gataaatgct	1440
tcaataatat	tgaaaaagga	agagtatgag	tattcaacat	ttccgtgtcg	cccttattcc	1500
ctTTTTtgcg	gcattttgcc	ttcctgtttt	tgctcaccca	gaaacgctgg	tgaaagtaaa	1560
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aagttgcagg	accacttctg	cgctcggccc	ttccggctgg	ctggtttatt	gctgataaat	2160
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ccgggttgga	ctcaagacga	taggtaccgg	ataaggcgca	gcggtcgggc	tgaacggggg	2820
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gcgagcattg	agaaagcgcc	acgcttcccc	aaggagaaaa	ggcggacagg	tatccggtaa	2940

PA041WO_sequence_listing.ST25.txt

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cggaatt 3607

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<210> 31
<211> 6
<212> PRT
<213> Artificial Sequence

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<220>
<223> N-terminal linker

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<400> 31

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Cys Gly Asp Glu Gly Gly
1          5

```

```

<210> 32
<211> 6
<212> PRT
<213> Artificial Sequence

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```

<220>
<223> C-terminal linker

```

```

<400> 32

```

```

Gly Gly Glu Asp Gly Cys
1          5

```

```

<210> 33
<211> 5
<212> PRT
<213> Artificial Sequence

```

```

<220>
<223> Linker

```

```

<400> 33

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```

Gly Gly Lys Gly Gly
1          5

```

```

<210> 34

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PA041WO_sequence_listing.ST25.txt

<211> 3
 <212> PRT
 <213> Artificial Sequence

<220>
 <223> N-terminal glycine linker

<220>
 <221> REPEAT
 <222> (1)..(1)
 <223> Glycine can be repeated from zero to five times

<220>
 <221> REPEAT
 <222> (3)..(3)
 <223> Glycine can be repeated from zero to twelve times

<400> 34

Gly Cys Gly
 1

<210> 35
 <211> 9
 <212> PRT
 <213> Artificial Sequence

<220>
 <223> N terminal glycine serine linkers

<220>
 <221> REPEAT
 <222> (1)..(1)
 <223> Glycine can be repeated from zero to five times

<220>
 <221> REPEAT
 <222> (3)..(3)
 <223> Glycine can be repeated from zero to ten times

<220>
 <221> REPEAT
 <222> (4)..(4)
 <223> Serine can be repeated from zero to two times

<220>
 <221> REPEAT
 <222> (5)..(9)
 <223> These residues can be repeated from zero to three times as a group

<400> 35

Gly Cys Gly Ser Gly Gly Gly Ser
 1 5

<210> 36
 <211> 3
 <212> PRT
 <213> Artificial Sequence

<220>
 <223> C-terminal glycine linker

PA041WO_sequence_listing.ST25.txt

<220>
 <221> REPEAT
 <222> (1)..(1)
 <223> Glycine can be repeated from zero to twelve times

<220>
 <221> REPEAT
 <222> (3)..(3)
 <223> Glycine can be repeated from zero to five times

<400> 36

Gly Cys Gly
 1

<210> 37
 <211> 10
 <212> PRT
 <213> Artificial Sequence

<220>
 <223> C terminal glycine serine linkers

<220>
 <221> REPEAT
 <222> (1)..(1)
 <223> Glycine can be repeated from zero to ten times

<220>
 <221> REPEAT
 <222> (2)..(2)
 <223> Serine can be repeated from zero to two times

<220>
 <221> REPEAT
 <222> (3)..(7)
 <223> These residues can be repeated from zero to three times as a group

<220>
 <221> REPEAT
 <222> (8)..(8)
 <223> Glycine can be repeated from zero to eight times

<220>
 <221> REPEAT
 <222> (10)..(10)
 <223> Glycine can be repeated from zero to five times

<400> 37

Gly Ser Gly Gly Gly Gly Ser Gly Cys Gly
 1 5 10

<210> 38
 <211> 5
 <212> PRT
 <213> Artificial Sequence

<220>
 <223> Glycine serine linker

<220>

PA041WO_sequence_listing.ST25.txt

<221> REPEAT
 <222> (1)..(5)
 <223> These residues can be repeated any times as a group

<400> 38

Gly Gly Gly Gly Ser
 1 5

<210> 39
 <211> 10
 <212> PRT
 <213> Artificial Sequence

<220>
 <223> N-terminal gamma1

<400> 39

Cys Gly Asp Lys Thr His Thr Ser Pro Pro
 1 5 10

<210> 40
 <211> 10
 <212> PRT
 <213> Artificial Sequence

<220>
 <223> C-terminal gamma 1

<400> 40

Asp Lys Thr His Thr Ser Pro Pro Cys Gly
 1 5 10

<210> 41
 <211> 17
 <212> PRT
 <213> Artificial Sequence

<220>
 <223> N-terminal gamma 3

<400> 41

Cys Gly Gly Pro Lys Pro Ser Thr Pro Pro Gly Ser Ser Gly Gly Ala
 1 5 10 15

Pro

<210> 42
 <211> 18
 <212> PRT
 <213> Artificial Sequence

<220>
 <223> C-terminal gamma 3

<400> 42

Pro Lys Pro Ser Thr Pro Pro Gly Ser Ser Gly Gly Ala Pro Gly Gly
 1 5 10 15

PA041WO_sequence_listing.ST25.txt

Cys Gly

<210> 43
 <211> 6
 <212> PRT
 <213> Artificial Sequence

 <220>
 <223> N-terminal glycine linker

 <400> 43

Gly Cys Gly Gly Gly Gly
 1 5

<210> 44
 <211> 6
 <212> PRT
 <213> Artificial Sequence

 <220>
 <223> C-terminal glycine linker

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 1 5

<210> 45
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Gly Gly Lys Lys Gly Cys
 1 5

<210> 46
 <211> 6
 <212> PRT
 <213> Artificial Sequence

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 <223> N-terminal glycine-lysine linker

 <400> 46

Cys Gly Lys Lys Gly Gly
 1 5

<210> 47
 <211> 4
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<223> C.terminal linker

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37

<210> 49

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<400> 49

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<223> oligonucleotide primer

<400> 51

cgatgcattt catccttagt tatcaatacg ctgggttcag

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<210> 52

<211> 36

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<213> Artificial Sequence

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<223> oligonucleotide primer

<400> 52

ggcaaaatta gagactgtta ctttaggtaa gatcgg

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<210> 56
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 <223> Lys-HBcAg(as) primer

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<210> 60
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 <212> DNA
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 <223> Lys-HBcAg(s) primer

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<210> 61
 <211> 38
 <212> DNA
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<220>
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<400> 61
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<210> 62
 <211> 37
 <212> DNA
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<212> DNA

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<223> HBcAg149hind-as

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<210> 66

<211> 33

<212> DNA

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<400> 66

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<210> 67

<211> 38

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<213> Artificial Sequence

<220>

<223> HBcAgwthindIIII primer

<400> 67

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<210> 68

<211> 10

<212> PRT

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<223> epitope CeH3

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<211> 51

<212> DNA

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<220>

<223> CeH3fwd primer

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gtc	51
val	

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<211> 17

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<213> Artificial Sequence

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<400> 70

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val

<210> 71

<211> 51

<212> DNA

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<220>

<223> CeH3rev primer

<400> 71

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<210> 72

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<223> CeH3rev primer peptide

<400> 72

Asp Glu Leu Asn Asn Gly Val
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<210> 73

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<212> DNA

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<223> HBcAg-wt EcoRI fwd primer

<400> 73

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<220>
 <223> HBcAg-wt Hind III rev primer

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38

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 <213> Mus musculus

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<210> 78
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<211> 30

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<213> Artificial Sequence

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<223> primer p1.46

<400> 81

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<210> 82

<211> 43

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<213> Artificial Sequence

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<223> primer p1.47

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43

<210> 83

<211> 43

<212> DNA

<213> Artificial Sequence

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<400> 83

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43

<210> 84

<211> 6

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<213> homo sapiens

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<210> 85

<211> 6

PA041WO_sequence_listing.ST25.txt

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 <213> *Xenopus laevis*

<400> 86

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<210> 87
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 <213> *Rattus norvegicus*

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<210> 88
 <211> 6
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 <213> *Cavia porcellus*

<400> 88

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<210> 89
 <211> 15
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<210> 90
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<400> 90

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<210> 91
 <211> 42
 <212> PRT
 <213> *Homo sapiens*

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<400> 91

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Gly Leu Met Val Gly Gly Val Val Ile Ala
 35 40

<210> 92

<211> 770

<212> PRT

<213> Homo sapiens

<400> 92

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Ala Leu Glu Val Pro Thr Asp Gly Asn Ala Gly Leu Leu Ala Glu Pro
 20 25 30

Gln Ile Ala Met Phe Cys Gly Arg Leu Asn Met His Met Asn Val Gln
 35 40 45

Asn Gly Lys Trp Asp Ser Asp Pro Ser Gly Thr Lys Thr Cys Ile Asp
 50 55 60

Thr Lys Glu Gly Ile Leu Gln Tyr Cys Gln Glu Val Tyr Pro Glu Leu
 65 70 75 80

Gln Ile Thr Asn Val Val Glu Ala Asn Gln Pro Val Thr Ile Gln Asn
 85 90 95

Trp Cys Lys Arg Gly Arg Lys Gln Cys Lys Thr His Pro His Phe Val
 100 105 110

Ile Pro Tyr Arg Cys Leu Val Gly Glu Phe Val Ser Asp Ala Leu Leu
 115 120 125

Val Pro Asp Lys Cys Lys Phe Leu His Gln Glu Arg Met Asp Val Cys
 130 135 140

Glu Thr His Leu His Trp His Thr Val Ala Lys Glu Thr Cys Ser Glu
 145 150 155 160

Lys Ser Thr Asn Leu His Asp Tyr Gly Met Leu Leu Pro Cys Gly Ile
 165 170 175

Asp Lys Phe Arg Gly Val Glu Phe Val Cys Cys Pro Leu Ala Glu Glu
 180 185 190

PA041WO_sequence_listing.ST25.txt

Ser Asp Asn Val Asp Ser Ala Asp Ala Glu Glu Asp Asp Ser Asp Val
 195 200 205
 Trp Trp Gly Gly Ala Asp Thr Asp Tyr Ala Asp Gly Ser Glu Asp Lys
 210 215 220
 Val Val Glu Val Ala Glu Glu Glu Glu Val Ala Glu Val Glu Glu Glu
 225 230 235 240
 Glu Ala Asp Asp Asp Glu Asp Asp Glu Asp Gly Asp Glu Val Glu Glu
 245 250 255
 Glu Ala Glu Glu Pro Tyr Glu Glu Ala Thr Glu Arg Thr Thr Ser Ile
 260 265 270
 Ala Thr Thr Thr Thr Thr Thr Thr Glu Ser Val Glu Glu Val Val Arg
 275 280 285
 Glu Val Cys Ser Glu Gln Ala Glu Thr Gly Pro Cys Arg Ala Met Ile
 290 295 300
 Ser Arg Trp Tyr Phe Asp Val Thr Glu Gly Lys Cys Ala Pro Phe Phe
 305 310 315 320
 Tyr Gly Gly Cys Gly Gly Asn Arg Asn Asn Phe Asp Thr Glu Glu Tyr
 325 330 335
 Cys Met Ala Val Cys Gly Ser Ala Met Ser Gln Ser Leu Leu Lys Thr
 340 345 350
 Thr Gln Glu Pro Leu Ala Arg Asp Pro Val Lys Leu Pro Thr Thr Ala
 355 360 365
 Ala Ser Thr Pro Asp Ala Val Asp Lys Tyr Leu Glu Thr Pro Gly Asp
 370 375 380
 Glu Asn Glu His Ala His Phe Gln Lys Ala Lys Glu Arg Leu Glu Ala
 385 390 395 400
 Lys His Arg Glu Arg Met Ser Gln Val Met Arg Glu Trp Glu Glu Ala
 405 410 415
 Glu Arg Gln Ala Lys Asn Leu Pro Lys Ala Asp Lys Lys Ala Val Ile
 420 425 430
 Gln His Phe Gln Glu Lys Val Glu Ser Leu Glu Gln Glu Ala Ala Asn
 435 440 445
 Glu Arg Gln Gln Leu Val Glu Thr His Met Ala Arg Val Glu Ala Met
 450 455 460

PA041WO_sequence_listing.ST25.txt

Leu Asn Asp Arg Arg Arg Leu Ala Leu Glu Asn Tyr Ile Thr Ala Leu
 465 470 475 480
 Gln Ala Val Pro Pro Arg Pro Arg His Val Phe Asn Met Leu Lys Lys
 485 490 495
 Tyr Val Arg Ala Glu Gln Lys Asp Arg Gln His Thr Leu Lys His Phe
 500 505 510
 Glu His Val Arg Met Val Asp Pro Lys Lys Ala Ala Gln Ile Arg Ser
 515 520 525
 Gln Val Met Thr His Leu Arg Val Ile Tyr Glu Arg Met Asn Gln Ser
 530 535 540
 Leu Ser Leu Leu Tyr Asn Val Pro Ala Val Ala Glu Glu Ile Gln Asp
 545 550 555 560
 Glu Val Asp Glu Leu Leu Gln Lys Glu Gln Asn Tyr Ser Asp Asp Val
 565 570 575
 Leu Ala Asn Met Ile Ser Glu Pro Arg Ile Ser Tyr Gly Asn Asp Ala
 580 585 590
 Leu Met Pro Ser Leu Thr Glu Thr Lys Thr Thr Val Glu Leu Leu Pro
 595 600 605
 Val Asn Gly Glu Phe Ser Leu Asp Asp Leu Gln Pro Trp His Ser Phe
 610 615 620
 Gly Ala Asp Ser Val Pro Ala Asn Thr Glu Asn Glu Val Glu Pro Val
 625 630 635 640
 Asp Ala Arg Pro Ala Ala Asp Arg Gly Leu Thr Thr Arg Pro Gly Ser
 645 650 655
 Gly Leu Thr Asn Ile Lys Thr Glu Glu Ile Ser Glu Val Lys Met Asp
 660 665 670
 Ala Glu Phe Arg His Asp Ser Gly Tyr Glu Val His His Gln Lys Leu
 675 680 685
 Val Phe Phe Ala Glu Asp Val Gly Ser Asn Lys Gly Ala Ile Ile Gly
 690 695 700
 Leu Met Val Gly Gly Val Val Ile Ala Thr Val Ile Val Ile Thr Leu
 705 710 715 720
 Val Met Leu Lys Lys Lys Gln Tyr Thr Ser Ile His His Gly Val Val
 725 730 735

PA041WO_sequence_listing.ST25.txt

Glu Val Asp Ala Val Thr Pro Glu Glu Arg His Leu Ser Lys Met
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Gln Gln Asn Gly Tyr Glu Asn Pro Thr Tyr Lys Phe Phe Glu Gln Met
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Gln Asn
 770

<210> 93
 <211> 82
 <212> PRT
 <213> Homo sapiens

<400> 93

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Lys Leu Val Phe Phe Ala Glu Asp Val Gly Ser Asn Lys Gly Ala Ile
 35 40 45

Ile Gly Leu Met Val Gly Gly Val Val Ile Ala Thr Val Ile Ile Ile
 50 55 60

Thr Leu Val Met Leu Lys Lys Gln Tyr Thr Ser Asn His His Gly Val
 65 70 75 80

Val Glu

INTERNATIONAL SEARCH REPORT

International Application No

PCT/EP 03/07864

A. CLASSIFICATION OF SUBJECT MATTER

IPC 7 A61K39/385 A61K39/00 A61P25/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 7 A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

EPO-Internal, BIOSIS, EMBASE, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 00/32227 A (CYTOS BIOTECHNOLOGY AG) 8 June 2000 (2000-06-08) page 82 - page 93	1-51
Y	----- page 82 - page 93	1-51
Y	WO 01/053457 A (UNIVERSITY OF CONNECTICUT HEALTH CENTER) 26 July 2001 (2001-07-26) page 41 - page 46	1-51
P, X	----- WO 02/056905 A (CYTOS BIOTECHNOLOGY AG) 25 July 2002 (2002-07-25) page 221 - page 254	1-51



Further documents are listed in the continuation of box C.



Patent family members are listed in annex.

° Special categories of cited documents:

A document defining the general state of the art which is not considered to be of particular relevance

E earlier document but published on or after the international filing date

L document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

O document referring to an oral disclosure, use, exhibition or other means

P document published prior to the international filing date but later than the priority date claimed

T later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

X document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

Y document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

S document member of the same patent family

Date of the actual completion of the international search

16 December 2003

Date of mailing of the international search report

07/01/2004

Name and mailing address of the ISA

European Patent Office, P.B. 5818 Patentlaan 2
NL - 2280 HV Rijswijk
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Fax: (+31-70) 340-3016

Authorized officer

Moreau, J

INTERNATIONAL SEARCH REPORT

International application No.
PCT/EP 03/07864

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

Although claims 46-49 are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.
2. ☐ Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:
3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/EP 03/07864

Patent document cited in search report		Publication date	Patent family member(s)	Publication date
WO 0032227	A	08-06-2000	AU 1402000 A	19-06-2000
			BR 9915771 A	26-12-2001
			CA 2354183 A1	08-06-2000
			CN 1333693 T	30-01-2002
			EP 1135162 A2	26-09-2001
			WO 0032227 A2	08-06-2000
			NZ 512456 A	31-10-2003
			PL 348452 A1	20-05-2002
			ZA 200105050 A	20-06-2002
WO 0153457	A	26-07-2001	AU 2959201 A	31-07-2001
			WO 0153457 A2	26-07-2001
WO 02056905	A	25-07-2002	CA 2433316 A1	25-07-2002
			CA 2433862 A1	25-07-2002
			EP 1353691 A2	22-10-2003
			WO 02056905 A2	25-07-2002
			WO 02056907 A2	25-07-2002
			US 2003175711 A1	18-09-2003
			US 2003175290 A1	18-09-2003
			WO 03031466 A2	17-04-2003
			WO 03039225 A2	15-05-2003
			WO 03040164 A2	15-05-2003
			US 2003157479 A1	21-08-2003